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The Response of the Central Nervous System to Experimental Colitis

by

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Abstract

The aims of this study were to examine the central nervous system (CNS) response to colitis by means of neuronal activation using Fos immunohistochemistry and activation of microglia. They were also to determine whether microglial activation is mediated by the vagus nerve or assisted by the central production of cytokines or chemotactic signals. The induction of colitis resulted in the activation of neurons and microglia (induced to express MHC class II antigen) in the area postrema. Vagotomy blocked the activation of microglia in response to colitis, suggesting that the vagus nerve might carry peripheral signals capable of activating microglia. In addition, the late activation of microglia was preceded by the immunoreactive expression of the chemokine monocyte chemotactic protein-1 and IL-1β in the area postrema and in other CNS structures. Taken together, these data suggest that microglia could be involved in the central processing of vagal mediated peripheral signals, and might actively participate in the CNS homeostatic regulation of peripheral immune responses.

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List of Abbreviations

ACTH:	Adrenocorticotrophic hormone
AP:	Area postrema
AVP:	Arginine vasopressin
BBB:	Blood-brain barrier
BDNF:	Brain derived neurotrophic factor
CD:	Crohn's disease
CNS:	Central nervous system
CRF:	Corticotrophin releasing factor
DMN:	Dorsal motor nucleus of the vagus
ENS:	Enteric nervous system
CVO:	Circumventricular organ
GFAP:	Glial fibrillary acidic protein
HPA:	Hypothalamic-pituitary-adrenal
IBD:	Inflammatory bowel disease
i.c.v.	intracerebroventricularly
IFN:	Interferon
IL:	Interleukin
i.p.:	intraperitoneal
IR:	Immunoreactive
LPS:	Lipopolysaccharide

MCP:	Monocyte chemotactic protein
MHC:	Major histocompatibility complex
MPO:	Myeloperoxidase
mRNA:	messenger Ribonucleic acid
NGF:	Nerve growth factor
NT-3:	Neurotrophin-3
NTS:	Nucleus of the solitary tract
OT:	Oxytocin
OVLT:	Organum vasculosum of the lamina terminalis
PG:	Prostaglandin
SD:	Standard deviation
TNBS:	Trinitrobenzene sulfonic acid
TGF:	Transforming growth factor
TNF:	Tumor necrosis factor
UC:	Ulcerative colitis

CHAPTER 1

INTRODUCTION

The last decade has provided extensive support for the relatively new concept of brain-immune system interactions. This has been divided into two avenues of research: one avenue has focused on the active participation of the brain in the homeostatic regulation of peripheral immune responses, and the other avenue has centered on the response of the CNS to peripheral immune challenge. This study has examined the effects of colonic inflammation on various physiological changes in the CNS. The following pages will review: a) inflammation, inflammatory bowel disease as a type of inflammation, and how hapten-induced colonic inflammation is a reliable model of peripheral inflammation; b) how brain-gut interactions occur during inflammatory bowel disease, and how neuronal and humoral signals travel between the periphery and the CNS and affect separately neuronal and glial activation and the production of cytokines; c) the homeostatic effects of cytokine expression within the CNS, and how microglia, which are the CNS resident immune cells respond to peripheral immune signals by producing cytokines; and d) circumventricular organs where blood-borne immune signals could easily enter the CNS and stimulate its response. The dorsal vagal complex, which receives both neuronal and blood-borne signals, will be proposed as the site of integration of the peripheral input. This will lead to the hypothesis that resident microglia in the dorsal vagal complex are activated following colonic inflammation.

1.1 The immunological basis of inflammation

Inflammation is generally a localized protective response which serves to destroy and wall-off both the deleterious agent and the damaged tissue (31). Among its classical signs are pain (dolor), heat (calor), redness (rubor), swelling tumor), and loss of function. Microscopically, it consists of (a) dilation of arterioles, capillaries, and venules, with increased permeability and blood flow; (b) exudation of fluids, including plasma proteins; and (c) leukocytic migration into the inflammatory site. Most forms of acute and chronic inflammation are amplified as well as propagated by the recruitment of humoral and cellular components of the immune system. Immunologically mediated elimination of foreign material proceeds through a series of integrated steps, which involve the binding of a recognition component of the immune system to the antigen and the subsequent activation of an amplification system, initiating the production of proinflammatory substances. The destruction of antigens by immune mechanisms is mediated by phagocytic cells. Such cells may migrate freely or may exist at fixed tissue sites as components of the mononuclear phagocyte system. Macrophages are central components of this system. Destruction of antigens outside of the mononuclear phagocyte system is generally mediated by polymorphonuclear leukocytes (neutrophils) which are recruited from circulating blood. In most instances, immune responses are ongoing and lead to the elimination of antigens without producing clinically detectable inflammation, however, upon encountering a large amount of antigen, antigen in an unusual

location, or antigen that was difficult to digest, inflammation becomes clinically apparent (31). A similar scenario occurs during the onset of inflammatory bowel disease.

1.2 Inflammatory bowel disease is a subtype of inflammation

Inflammatory bowel disease (IBD) is a chronic condition characterized by an unpredictable clinical course (22). Although a causative factor has not been identified, there is support for a number of cytokines as well as chemokines (87) in initiating and amplifying the inflammatory response. The two main forms of inflammatory bowel disease are Ulcerative colitis (UC) and Crohn's disease (CD) (51). They are usually characterized microscopically by mucosal and submucosal infiltration of polymorphonuclear leukocytes, macrophages, lymphocytes, connective tissue mast cells, and fibroblasts, and macroscopically by ulceration and thickening of the bowel wall. A previous study (72) has identified the hapten Trinitrobenzenesulfonic acid (TNBS) as a reliable inducer of colonic inflammation with characteristics similar to those apparent in Crohn's disease. The long duration of the hapten-induced inflammation, the composition of the inflammatory cell population, the minimal mechenical injury (compared to other chemical inducers of experimental colitis such as acetic acid or dextran sodium sulfate), and the ability to monitor the progression of the disease by a variety of methods make it a reliable model for the investigation of colonic inflammation (reviewed in ref.(90)).

Cytokines and chemokines have a key role in regulating the intestinal immune system. They are produced by a variety of immune cells such as lymphocytes, monocytes, intestinal macrophages, granulocytes, epithelial cells, endothelial cells, and fibroblasts. They have pro-inflammatory [interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), IL-6, IL-8, IL-12] or anti-inflammatory functions [interleukin-1 receptor antagonist (IL-1ra), IL-4, IL-10, transforming growth factor-β (TGF-β)]. Mucosal and systemic concentrations of many pro- and anti-inflammatory cytokines are increased in inflammatory bowel disease. There is an imbalance between the cytokine IL-1 and its antagonist IL-1ra, in patients with Crohn's disease, Ulcerative colitis, diverticulitis, and infectious colitis (2). In addition, the inhibition of pro-inflammatory cytokines or the administration of anti-inflammatory cytokines reduces inflammation in animal models, such as the dextran sulfate colitis, TNBS, or the genetically engineered model of IL-10 knockout mice (reviewed in Ref. 87).

Another group of molecules, which are in fact a subgroup of the cytokine family, is the chemokine group (65). This rather newly recognized group of small cytokines plays a major role in determining the cellular composition in the site of inflammation which may depend on both the nature of the secreted chemokines as well as the relative expression of specific cell surface receptors on different cell types. The existence of receptors with different affinities and cross reactive binding capabilities on each type of immunocyte make it possible to selectively attract specific subgroups of cells.

All of these inflammatory mediators are being produced during the onset, progression, and termination of all immune responses. As these molecules accumulate in the peripheral site of inflammation they enter blood vessels and are able to circulate through the body and reach other regions (like neighboring lymph nodes and the brain). Some of these regions could respond to these signals and actively participate in the regulation and/or modulation of these events (the effect of some these circulating cytokines will be discussed later).

1.3 Brain-gut interactions and their relation to inflammatory bowel disease

What are the neuronal projections between the nervous system and the site of inflammation, in our case the viscera? The enteric nervous system innervates the gastrointestinal tract, the pancreas, and the gall bladder. It consists of local sensory neurons that record alterations in the tension of the gut walls and the chemical environment, as well as interneurons and motor neurons that control the muscles of the gut wall and vasculature and the secretory activity of the mucosa. Hence, the enteric nervous system can function autonomously, although, its activity is normally regulated by CNS reflexes (30;106).

The enteric nervous system is regulated by extrinsic innervation that is supplied by the parasympathetic and sympathetic systems. Parasympathetic preganglionic neurons project directly to enteric ganglia of the stomach, colon,

and rectum through the vagus and the pelvic nerves. The postganglionic sympathetic innervation of the gastrointestinal tract is primarily from prevertebral sympathetic ganglia, with some innervation from the cervical paravertebral chain. The innervation of the gut by the sympathetic and parasympathetic fibers of the autonomic nervous system provides a second level of control of motility and secretion, but also can outweigh intrinsic enteric activity in situations of emergency or stress (30;106).

The output of the autonomic nervous system is influenced by a large number of brain regions: the cerebral cortex, the hippocampus, the entorhinal cortex, parts of the thalamus, and the basal ganglia. Most of these regions produce their actions by way of the hypothalamus (62). The hypothalamus in turn integrates the information it receives into a coherent pattern of autonomic response. Although the hypothalamus exerts a major overall control over the autonomic nervous system, many autonomic functions do not require continuous monitoring by the hypothalamus, and can be coordinated by nuclei in the brainstem. The major coordinating center for autonomic function in the brain stem is the *nucleus of the solitary tract* (NTS). This nucleus receives sensory information from most organs of the body, and then uses this information to modulate autonomic function in two ways. First, the nucleus controls simple autonomic function by means of a set of reflex circuits. Sensory visceral afferent fibers from the heart, lungs, and gastrointestinal tract project to specific subnuclei within the NTS in a viscerotopic fashion. These neurons project to

lower brainstem nuclei that connect to autonomic motor neurons controlling effectors. Second, the nucleus coordinates intricate homeostatic adjustments by transmitting information from autonomic targets to both higher and lower brain regions. These regions then relay integrated information required for complex autonomic control back to the NTS. Specifically, visceral afferents from an array of autonomic targets terminate in a conjoint region of the NTS called the *commissural nucleus,* which in turn projects to a wide range of brain stem and forebrain nuclei, including the amygdala, the paraventricular hypothalamic nucleus, and the bed nucleus of the stria terminalis. These nuclei then project back to the NTS, as well as other lower brain stem nuclei. In addition, these brain stem nuclei project directly to the autonomic output nuclei for the gastrointestinal system, mainly to the dorsal vagal nucleus and sympathetic preganglionic nuclei (63).

Parasympathetic preganglionic neurons within both the brainstem and the spinal cord project to postganglionic neurons in ganglia that are close to visceral targets or actually embedded in them. The axons of motor neurons in the dorsal vagal nucleus project in the vagus nerve to postganglionic neurons embedded in thoracic and abdominal targets- the lungs, esophagus, stomach, and upper intestinal tract. The axons of spinal parasympathetic preganglionic neurons leave the spinal cord via the ventral roots and project in the pelvic nerve to parasympathetic postganglionic neurons in the pelvic ganglion plexus. Pelvic ganglion neurons innervate the descending colon, bladder, and external

genitalia. Hence, visceral sensory input that is processed and relayed back to the NTS could project back to the gastrointestinal tract via either the dorsal vagal nucleus or the pelvic ganglion neurons (17).

Initial reports of enteric nervous system (ENS) involvement in intestinal inflammation came from observations of neural hyperplasia in Crohn's disease (CD) and colitis (95). Moreover, axonal necrosis, and ganglion cell degeneration are known as features of IBD (94). Specific neurotransmitters appear to either increase or decrease in CD and colitis (92). Other studies have suggested that the neuropeptide substance P is involved in the initiation of colitis (6;52;77;92). Primary afferent nerves are a key source of substance P in the gastrointestinal tract. A population of these nerves is sensitive to the neurotoxin capsaicin (43). Ablation of these primary afferent, capsaicin-sensitive nerves by systemic administration of capsaicin exacerbates inflammatory conditions (25;69). Accompanying the involvement of primary afferent nerves in gastrointestinal inflammation, studies have implicated a role for sympathetic nerves in colitis (69), as well as in joint inflammation (4). Rectal biopsies of patients with colitis, reveal an increase in mucosal adrenergic terminals (55;78). Another study have demonstrated the attenuation of colitis by sympathectomy and is probably the most striking support for the contribution of sympathetic nerves to the pathogenesis of colitis (69).

Other studies examined the effectiveness of lidocaine in ameliorating TNBS-induced colitis in the rat (68). The local anesthetic action of the sodium

channel blocker lidocaine can occur in three sites: primary afferent nerves, sympathetic nerves, or the enteric nervous system. In the absence of primary afferent nerves the effectiveness of lidocaine was partially reduced, implicating these nerves as one of the sites of action of the drug. The inability of this drug to worsen colitis, under these conditions suggested that lidocaine does not counteract the protective feature of primary afferent nerves, and that it must also be acting on either the sympathetic nerves or the enteric nervous system. In the absence of sympathetic nerves, lidocaine was more effective in attenuating TNBS-induced colitis, implying that lidocaine is acting on enteric nerves and that these nerves have a net pro-inflammatory role in colitis. The combination of removal of the sympathetic nerves by chemical sympathectomy and inhibition of the release of transmitter from enteric nerves, in the presence of primary afferents, results in the greatest attenuation of colitis.

In summary, previous data suggest that primary afferent, sympathetic and enteric nerves may promote the pathogenesis of colitis. Primary afferent capsaicin-sensitive nerves seem to have a protective role, since ablation of these nerves increased the severity of colitis. The beneficial effects of lidocaine in the TNBS-induced colitis model are likely to be due primarily to extrinsic capsaicin-sensitive afferent nerves or sympathetic nerves. There is also a possibility of an interaction between extrinsic and intrinsic nerves through as yet unknown mechanism.

1.4 CNS response to peripheral inflammation

As soon as peripheral inflammation occurs, the brain 'senses' the ongoing events by two major information pathways: one is neuronal and the other humoral. Neuronal signals travel from the periphery to the brain via afferent sensory projections that terminate in the NTS. Thereafter, these signals could either be transferred to higher brain centers via the thalamus or reach the parabrachial nucleus which projects to the hypothalamus and frequently results in the activation of the hypothalamic-pituitary-adrenal axis (HPA) (99).

The other exchange of information, between the brain and immune-challenged periphery, is humoral. As most of the CNS is surrounded by a blood-brain-barrier (BBB-to be discussed in detail later) which blocks the transition of cells and cytokines from blood circulation, this interaction occurs in regions that lack a BBB and are collectively termed circumventricular organs (CVO).

The CNS responds either by neuronal or glial (microglia, astroglia, and oligodendrocytes) activation. This has an important impact on the outcome of the physiological changes that follow, as different cell types vary in their capacity to process incoming signals and produce potent response signals. Both the neural input, which reaches the brainstem, and humoral (blood-borne) signals, which could activate neurons and glia (especially microglia and astrocytes) in the vicinity of CVOs, can induce the activation of the HPA axis.

1.4.1 The neuronal pathway of communication between the CNS and the periphery

1.4.1.1 The role of the vagus nerve in periphery-to-brain communication.

Peripheral inflammatory stimuli can induce the synthesis and release of various cytokines such as IL-1β. This results in a diverse number of CNS responses. The majority of propositions aimed to explain how peripheral cytokines affect the CNS have centered on the humoral pathway of communication. In contrast, some recent investigations (38;39;58) have switched to the neuronal periphery-to-brain signaling pathway, and focused primarily on the vagus nerve. In these studies, IL-1β has been shown to activate vagal afferent fibers that terminate in the NTS. Among the resultant effects were hyperalgesia, fever, anorexia, taste aversions, increased levels of plasma corticosteroid, and changes in brain norepinephrine levels (14;88).

According to previous reports, peripheral immune stimulation results in the peripheral and central (in the CNS) production of IL-1β. Blockade of the centrally expressed pro-inflammatory cytokine by its receptor antagonist (administered centrally) or sectioning of the vagus nerve resulted in inhibition of the behavioral symptoms (9;14). This led another group to determine the connection between centrally expressed IL-1β and the vagus nerve. Upon peripheral administration (i.p.) of either LPS (58) or IL-1β (39) to vagotomized and sham operated rats, vagotomy blocked the induction of IL-1β in the

corresponding brains. This has provided strong evidence for the role of the vagus nerve in transmitting cytokine signals from the periphery to the brain and inducing brain cytokines, all of which result in centrally regulated symptoms of the acute host defense response.

1.4.1.2 Activation of neurons along the hypothalamic-pituitary-adrenal axis

It is well established that stimulation of the immune system results in the activation of the HPA axis. Various models of immune stimulation have demonstrated the activation of neurons that constitute the autonomic and endocrine components of the paraventricular hypothalamic nucleus (PVH). One study used intravenous administration of lipopolysaccharide to induce an acute phase immune response and then neuroanatomical techniques to assess the subsequent activation of neurons (23). Immunohistochemistry for Fos (a common marker of neuronal activation) was coupled with retrograde transport of the neuroanatomical tracer Cholera toxin-b, which was injected into the vicinity of the PVH. Many of the activated (Fos expressing) cells projected to a number of anatomical structures, such as the visceromotor cortex, median preoptic nucleus, ventromedial preoptic area, bed nucleus of the stria terminalis, parabrachial nucleus, ventrolateral medulla, and the NTS, all of which extend to the paraventricular nucleus of the hypothalamus. This provided support for the existence of a neuroanatomical circuit, which connects the NTS (that carries

neural input from the periphery) to the paraventricular nucleus of the hypothalamus and feeds back to the periphery by means of hormone release.

Further studies have attempted to characterize the hypothalamic products that appear upon immune stimulation (56;97). Two of these are corticotropin-releasing factor (CRF) and vasopressin (AVP) which both play a dominant role in the increased plasma adrenocorticotrophic hormone (ACTH) levels following peripheral inflammation. A current model of HPA axis activation suggests that peripheral immune response increases circulating levels of cytokines. These could directly modulate the HPA axis response to immune activation, albeit their most potent effect is the induction of the release of prostaglandins (PG). The increase in prostaglandin levels leads to the activation of CRF-dependent pathways, together with CRF secretion from nerve terminals. PG release is an obligatory step for the activation of the HPA axis as indicated by the reduction of cytokine-mediated glucocorticoid release subsequent to the administration of PG blockers, while cytokine levels remain increased.

Finally, two previous studies have examined the role of the vagus nerve in mediating the immune stimulation of the HPA axis (detected by the release of adrenocoticotropin – ACTH). In one study, subdiaphragmatic vagotomy suppressed both the activation of CRF neurons and plasma ACTH which normally occur following endotoxin-induced inflammation (32). Vagotomy has also attenuated ACTH secretion normally stimulated by the administration peripheral Interleukin-1β (48). Taken together, both studies have illustrated the

involvement of vagal afferents in mediating immune stimulated activation of the HPA axis. Inhibition of the HPA axis activation, by vagotomy, was shown to be specific to immune stimulation by Interleukin-1β.

1.4.2 The humoral pathway of periphery to brain signaling

1.4.2.1 Neuronal activation by peripheral immune stimulation

The brain is 'wrapped' in a structure referred to as the blood-brain barrier (described in section 1.6). This makes it quite hard if not virtually impossible for cells or proteins to cross from the blood into the CNS. However, a number of CNS regions lack this structure and are termed circumventricular organs. Neurons that lie within these regions are more likely to be exposed to a large number of signals, which accompany peripheral immune stimulation, and for this reason they became the focus of several previous studies. To localize brain neurons involved in the acute phase of an immune response, systemic administration of bacterial endotoxin was performed in rat brains that were later examined immunohistochemically for the expression of Fos (a marker of neuronal activation) (85). Regions such as the organum vasculosum of the lamina terminalis (OVLT), the subfornical organ, and the area postrema, were all characterized by an increase in nuclear immunostaining, indicative of neuronal activation.

Humoral activation of neurons in circumventricular organs has a

significant impact on the outcome of an acute phase response. For example, the area postrema has been shown to project to the hypothalamus (57). Lesions in this area resulted in a significant attenuation of IL-1β-induced norepinephrine release in the hypothalamic paraventricular nucleus (45). The release of norepinephrine in the paraventricular nucleus of the hypothalamus is necessary for the proper activation of the neuroendocrine system (comprising of the HPA axis). Hence, the effect of humoral signals on neuronal activation in circumventricular organs could have a role in mediating the activation of the HPA axis.

1.4.2.2 Microglial activation following peripheral administration of endotoxin

The response of the CNS to peripheral signals conveyed by the humoral pathway is not necessarily confined to activation of neurons. The production of pro-inflammatory cytokines following administration of LPS is not restricted to the periphery, but also occurs in the brain. Microglial cells, the resident macrophages of the brain (discussed in the next section), have been shown to produce IL-1β mRNA (15) as well as protein (102), after peripheral administration of endotoxin. Microglia are regarded as immunologically quiescent under normal, non-pathological conditions (19). A variety of CNS injuries or infections results in the progressive conversion of the resting/ramified

microglia (so named due to their ramified processes) into active macrophages. A prior study observed microglial activation in the LPS model of peripheral inflammation (16). Activation of microglial cells throughout the CNS was apparent and peaked 24h following the administration of the endotoxin. Morphological transitions of the resting ramified microglia to round macrophage-like cells were present at multiple brain sites such as the anterior hypothalamus, thalamus, and the brainstem. Moreover, the endotoxin induced an increase in MHC class II antigen expression, which was maximal at day 3. At 7 days following treatment, microglia re-established their ramified shape (i.e., with processes extending into the surrounding neuropil) and the extent of MHC class II expression returned to control levels. Notwithstanding the presence of a large number of activated microglia in the rat brains, no sign of tissue damage were detected.

In conclusion, peripheral immune challenge leads to a substantial transient activation of microglial cells in the CNS. The activation of microglia is manifested morphologically by a shift from a ramified shape to a round/ameboid shape, and by an increase in the expression of MHC class II antigen. The functional significance of these morphological and histochemical changes is yet to be determined.

1.4.3 The role of cytokine expression within the CNS.

Some aspects of CNS response to peripheral inflammation are

demonstrated by intrinsic CNS production of various cytokines. Unlike in the periphery, where cytokines orchestrate the onset, progression, and termination of immune responses, the central production of these powerful molecules does not regularly lead to a central immune response (an immune response within the CNS), but often eventuates into subtle physiological changes that have a crucial impact on the regulation and maintenance of homeostasis. In fact, the expression and secretion of some cytokines within the CNS have been shown to be involved in the intricate autonomic, neuroendocrine, metabolic, and behavioral responses to peripheral inflammation.

A body of evidence has revealed the constitutive expression of genes encoding for a variety of cytokines and their receptors in the brain, implying that certain cytokines may assist in the normal functioning of the CNS (107;109). In addition, peripheral cytokines can induce the synthesis of cytokines within the CNS. Following peripheral administration of LPS, the expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is initially induced in perivascular cells, meningeal cells, and neurons in circumventricular organs and ventral surface of the medulla (13). Thereafter, TNF-α mRNA is induced in the paraventricular and arcuate hypothalamic nuclei and the NTS (7). Other groups have shown that during the course of peripheral inflammation the gene encoding for Interleukin-1β is similarly expressed in elevated amounts in the brain first in circumventricular organs, such as the subfornical organ, and then inside brain parenchyma in regions with an intact BBB, like the paraventricular and arcuate

nuclei of the hypothalamus (82).

A rather new concept suggests that the central action of cytokines is involved in regulating key factors of the CNS response to peripheral inflammation. It has corroborated the finding that those peripheral responses can be attenuated if not abolished by the central administration of Interleukin-1 receptor antagonist. The induction of the corticotropin-releasing hormone (CRH) gene, in the paraventricular nucleus of the hypothalamus in response to peripheral endotoxin administration, can be abrogated by central (yet not peripheral) administration of IL-1 receptor antagonist (47). This implies that the actions of IL-1 inside the brain are necessary for the induction of CRF mRNA during peripheral inflammation. In the same way, in mice, the central or peripheral administration of IL-1β generates a reduction in social behavior and loss of body weight which can be attenuated by pretreatment with central (i.c.v.) administration of Interleukin-1 receptor antagonist, suggesting that the outcome of peripheral IL-1β is centrally mediated (9). Nonetheless, blocking central IL-1β does not entirely eliminate the effects of peripheral endotoxin on social behavior in rats. Mice deficient for IL-1 receptor type I do not respond to central IL-1β, but retain their sensitivity to the central effects of (peripherally administered) LPS on social behavior. This is due in part to tumor necrosis factor-α, because pretreatment with intracerebroventricular TNF-α binding protein (that block this cytokine's activity) revokes the depressing effects of peripheral endotoxin on social behavior in IL-1 receptor I deficient mice (11). In another more recent

study, activation of the HPA axis (as measured by an increase in plasma adrenocorticotropin) by either central administration of TNF-α or local inflammation (in a turpentine model) was reduced by pretreatment with either TNF-α antiserum or the soluble TNF receptor construct (98). This suggested that the central action of this cytokine is also involved in the activation of the endocrine pathway. Mice deficient in IL-6 are less sensitive to the depressing effects of both IL-1 and LPS on social behavior when these substances are administered either peripherally or centrally, and their decreased sensitivity is completely due to IL-1, as pretreatment with central receptor antagonist abolishes the depressing effects of LPS in IL-6 deficient mice (reviewed in ref.(59)). Hence, an intricate repertoire of cytokines including IL-1, IL-6, and TNF-α is expressed in the CNS to regulate the central effect of peripheral inflammatory mediators.

1.5 Microglia- The brain's immune cells.

1.5.1 General role

Microglia are a class of mononuclear phagocytes intrinsic to the CNS and are the prime immune effector component of the brain (96). Although microglia do not cause disease, they respond to it and eventually assist in determining the pattern and rate of CNS recovery of function (34) through the secretion of bioactive agents including cytotoxins. Reactive microglia appear in almost every

type of CNS disorder, including infection, trauma, stroke, degeneration, and demyelination. As inflammatory cells, they are thought to be the major source of CNS- derived cytokines, help to regulate wound healing in neural tissues, and serve as an important link between systemic immune responses and the CNS. Furthermore, microglia produce a wide variety of cytotoxic agents, some of which demonstrate potent neurotoxic effects (18).

All experimental data indicates that the CNS is subject to active immune surveillance. Activation of the surveillant immune cell means it has been recently primed by an encounter with a foreign antigen. Of all glial cells in the CNS, microglia seem to be the most promptly inducible and efficient antigen-presenting cells.

1.5.2 Microglial activation

Activated microglia are commonly viewed as the principal source of various cytokines (IL-1, IL-6, TNF-α, colony stimulating factor, TGF-β, and various chemokines). Microglia respond to these cytokines by changes which include the following (110):

1. Regulation of proliferation and differentiation.
2. Induction, enhancement, and inhibition of the expression of cell surface antigens (MHC class I, II).
3. Stimulation of the secretion of other cytokines.

In the event of blood-brain barrier damage, the probability of T cell infiltration into the CNS, increases. Secretion of Interferon-γ by infiltrating activated T cells may be the initiating signal for microglial activation, by inducing microglia to express class I and II MHC antigens which primes these cells for subsequent cytokine production (91). In addition, infiltrating activated macrophages produce cytokines such as IL-1β, TNF-α, and IL-6, which in conjunction with IFN-γ could trigger microglia to produce their own cytokines (71). In summary, microglia form a network of resident immune effector cells of the CNS. Microglial activation occurs after injury or changes in the microenvironment, either before or without observable changes in other cell types. The mechanism by which microglial activation and proliferation are regulated *in vivo*, however, is still unknown.

1.6 What is the blood-brain barrier and how is it involved in brain-immune interactions?

The blood-brain barrier is an intricate structure comprised of different cell types. It represents the interface between the blood that supplies nutrients to the brain, and the brain that requires homeostasis of its environment to function properly (46). The barrier separates plasma from brain interstitial fluid. It includes the vascular-glial complex within brain tissue (endothelial blood-brain barrier) on one side, and the choroid plexus epithelium, the tanycytes in the circumventricular organs, and the arachnoid barrier (blood-cerebrospinal fluid

barrier) on the other (Figure 1.1). The circumventricular organs include the hypothalamic median eminence, the pituitary, choroid plexus, pineal gland, subfornical organ, subcommissural organ, OVLT, and the area postrema (46). In these regions, most of the capillaries are fenestrated. There are many vesicles in the cytoplasm and these structures are thought to transfer their contents across the cell. These structural features account for the enhanced transport across these cells.

Why are these regions not protected by the blood-brain barrier? In the pituitary, the blood-brain barrier seems to be absent because neurosecretory products have to pass into the circulation. In the subfornical organ, a chemoreceptive area, the transcellular transport is required for water balance and other homeostatic functions. These leaky regions are isolated from the rest of the brain by specialized ependymal cells (tanycytes) that line the structures located along the ventricular surface close to the midline. Large substances such as cytokines or the bacterial product lipopolysaccharide are generally unable to cross from the bloodstream to the brain. At any time, cytokine entry or immune cell infiltration into the brain is more probable through these regions, and thus they are considered a hot spot for brain-immune cell interaction.

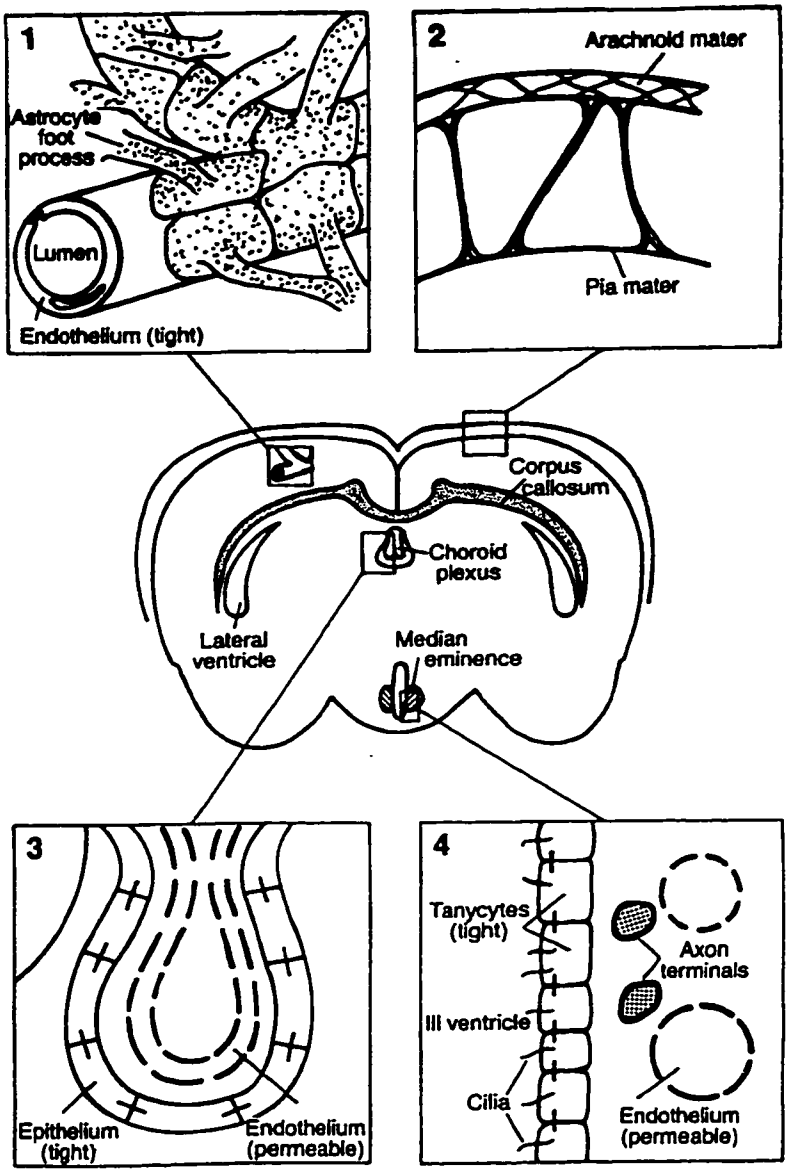

FIGURE 1.1

Diagrammatic representation of the blood-brain barrier and the blood-cerebrospinal fluid barrier in circumventricular organs such as the median eminence. Taken from Ref. (50).

1.7 Summary

Colonic inflammation is characterized both by immunological and neuronal features. Colitis is accompanied by the activation of many immune system cellular components such as macrophages, neutrophils, and T-cells. All these depend on the proper production and secretion of pro- and anti-inflammatory cytokines as well as chemokines. The intricate extrinsic and intrinsic innervation of the gut are additional participants in the initiation and progression of the inflammatory conditions of colitis (92), as seen in the activation of peripheral enteric nerves, and as demonstrated in sympathectomized animals (69). The interplay between neuronal and humoral aspects in chronic inflammation, such as colitis, make it a convenient model for the study of brain-immune interaction for two reasons: visceral inflammation is far from the CNS, and it does not directly expose the brain to an immunological insult, and the extensive innervation of the gut as well as the neuronal projections from the viscera to the brain (and vice versa) allow us to locate anatomical sites of the brain that could be involved in the regulation of visceral inflammation.

Similar to other models of peripheral inflammation, the hapten-induced model of colitis generates peripheral neuronal and humoral signals, which could be conveyed to the CNS through vagal afferents and circumventricular organs respectively. The dorsal vagal complex, which receives both neuronal (through the NTS) and humoral (through the fourth ventricle) signals, could serve as a

site of integration. Due to their immunological competence, microglia are suggested as the prime cell-type of the brain, capable of responding to the signals generated by an acute peripheral immune response following hapten-induced colitis.

On the basis of the information presented above, I propose the following hypothesis:

Hypothesis

Colitis is followed by the initiation of neuronal and circulating blood-borne signals, all of which reach the dorsal vagal complex of the brainstem and trigger the activation of microglia.

From this, a series of objectives have been addressed:

Objectives

1. Examine CNS response to colitis by means of neuronal activation (using c-fos expression as a marker of neuronal activation).
2. Demonstrate the activation of microglia in response to colitis.
3. Establish whether microglial activation, in response to colitis, is mediated either by a neural route of communication or a blood-borne route.
4. Determine whether the activation of microglia is preceded by the expression of chemotactic signals (In specific, the chemokine monocyte chemotctic

protein-1 (MCP-1)).

5. Examine the production of the cytokine Interleukin-1β in the region where microglial activation occurs, upon the induction of colitis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Male Wistar rats (150-200 g) were maintained under controlled environmental conditions (23-24°C, regular rat chow and water ad libitum) for at least 5 days before the experiment.

2.2 Induction of Colitis

Colitis was induced by a method similar to Morris et al. (72). 0.5 mL of TNBS (60 mg/mL) dissolved in 50% ethanol (v/v) was instilled into the lumen of the colon through a polyethylene catheter inserted rectally such that the tip was approximately 7 cm proximal to the anus. The catheter used for the administration of TNBS was not used for any other intracolonic administration.

2.3 Animal perfusion and tissue preparation

A laparotomy was carried out in animals anesthetized with pentobarbitol and the involved distal colon removed. The collected segment of colon was opened longitudinally along the mesenteric border in phosphate buffered- saline (PBS; pH 7.4), stretched on Sylgard and pinned flat as previously described (76). After tissue sampling, each rat was immediately perfused intracardially with 200 ml of cold saline (0.9% NaCl) followed by 250 ml of cold 4% paraformaldehyde (pH 7.4) in PBS (0.1 M). Brains were removed and post-fixed overnight in the same fixative at 4°C. After rinses (3x10min) in phosphate buffered saline (PBS), fixed brains were transferred to 20% sucrose-buffered

solution and stored at 4°C overnight. In preparation for sectioning, brains were embedded in OCT embedding medium (Sakura Finetek Inc., Torrance, CA, USA). Consecutive frozen coronal sections (30 μm thickness) of anterior brain (0.0 to –4.5mm, Bregma) and brainstem (-15 to –15.3mm, Bregma) were cut and collected in PBS.

2.4 Assessment of Colonic Damage

The severity of colitis was assessed in three ways: macroscopic damage scoring, histological damage scoring and colonic myeloperoxidase (MPO) activity assay. In addition, the change in body weight was recorded over the course of each study. Following sacrifice, the distal colon of each rat was removed and examined macroscopically. The criteria for scoring of macroscopic damage has previously been described (68) and is outlined in Table 2.1 (adopted from ref.(72)). Colonic damage was scored by the same individual and under 'blind' conditions, to minimize observer bias. After the macroscopic scoring, the segment of colon was dissected into two grossly identical pieces: the first piece was used for measurement of MPO activity, and the second for histological evaluation. MPO activity as a quantitative index of inflammation (68), was determined using an assay described by Krawisz et al. (53).

TABLE 2.1 Criteria for Macroscopic scoring of Colonic Damage

Feature	Score
Ulceration	
Normal appearance	0
Focal hyperemia, no ulcers	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at 1 site	3
2 or more sites of ulceration and inflammation	4
Major sites of damage extending > 2 cm along length of colon, score is increased by 1 for each additional cm of involvement	6-10
	plus
Adhesions	
No adhesions	0
Minor adhesions (colon can be easily separated from other tissue)	1
Major adhesions	2
Diarrhea	
No	0
Yes	1
Thickness	
Maximal bowel wall thickness (x), in mm, was added to above score	x
	Total score

Colonic samples for histology were fixed overnight by immersion in a solution containing 1% formalin, 1% glacial acetic acid and 80% methanol at 4°C, dehydrated, embedded in paraffin, cut into 12μm thickness and stained with haematoxylin and eosin. Histological scoring was based on a semi-quantitative scoring system in which the following features were considered and scored as follows: extent of destruction of normal mucosal architecture (0, normal; 3, maximal damage), presence and degree of cellular infiltration (0, normal; 3, maximal infiltration), extent of muscle thickening (0, normal; 3, maximal thickness), presence or absence of crypt abscesses (0, absent; 1, present) and the presence or absence of goblet cell mucus depletion (0, absent; 1, present). In each case a numerical score was given with a maximum score of 11. All histological assessments and cell counts were performed using coded slides to minimize observer bias. To control for the chemical damage caused by ethanol and TNBS, which is not related to the inflammation, one group of rats (TNBS 72 h) was treated with the anti-inflammatory glucocorticoid dexamethasone (2mg/kg; Vetoquinol Canada Inc.) a day prior to TNBS treatment.

2.5 Subdiaphragmatic Vagotomy

A standard operating procedure for chronic subdiaphragmatic vagotomy previously described by Powley et al. (81) was employed. Male Wistar rats (100-120g) were placed on a liquid diet (Isocal, Mead Johnson) available ad

libitum for a minimum of 7 days prior to surgery, which is followed by a loss of the rats capacity to digest solid food. This allowed the animals to adapt to a novel liquid diet prior to surgery. Anesthesia was induced with halothane 5%, reduced to a continuous level 1.5%, and the stomach exposed by a midline incision. The liver was gently dissected away from the stomach to expose the esophagus and the vagal branches were identified with a dissecting microscope. All visible branches of the vagus were then ligated (5-0 Silk) and cut between ligatures using a cauterizing forceps. Postoperatively, all animals were treated with antibiotics (Gentamicin;0.4ml/100g) and cherry flavored Tylenol (1200mg/Kg) was added to their drinking water. After a 24h fasting period, rats were maintained on the liquid diet as explained above for at least 10 days until the terminal experiments. To assess vagal integrity following vagotomy, the animals were injected (i.p.) with the retrograde fluorescent tracer Fast Blue (1mg/150g;Sigma) three days prior to the terminal perfusion. After sectioning, fluorescent microscopy was used to determine the presence of Fast Blue in the area postrema which was an indicator of the successful uptake of the tracer. Afferent denervation was verified by the lack of fluorescent labeling of the nodose ganglia and vagal efferent denervation was confirmed by the lack of fluorescently labeled cells in the dorsal motor nucleus of the vagus.

2.6 Immunohistochemistry

Coronal sections of anterior brain and brainstem were repeatedly (x3)

rinsed in PBS containing 0.1% Triton X-100 (PBST) and incubated for 48 h at 4C with one of the following primary antibodies: The antibody OX-42, which binds to the complement receptor type 3 (CD11b) (86), was used (1:500) to visualize macrophages and microglia. Astrocytes were visualized with an antibody against glial fibrillary acidic protein (GFAP)(1:250;bti). Major histocompatibility complex (MHC) class II was detected with the OX-6 antibody (1:500; Serotec). Fos protein was labeled with a polyclonal antibody (1:2000;Oncogene Science). The chemokine monocyte chemotactic protein-1 (MCP-1) was stained with a rabbit polyclonal antibody directed against rat MCP-1 (1:100; Serotec). After incubation with primary antibodies, sections were washed (3X 10min) with PBST and incubated for 1h at room temperature with either Donkey anti-mouse immunoglobulin (IgG) or Donkey anti-rabbit conjugated to CY3 at a dilution of 1:100. Subsequent to further PBST washing (3 x 10 min), sections were mounted in bicarbonate-buffered glycerol (pH 8.6). To control for secondary non-specific staining samples were incubated in antibody diluent and then processed identically with the respectable secondary antibody. The preparations were then examined under a Zeiss Axioplan fluorescence microscope and photographed using Kodak Tmax film.

Labeling of the cytokine Interleukin-1β was performed in a manner of tissue preparation and incubation modified from the above and similar to a protocol provided by the manufacturer of the cytokine specific antibody (R&D Systems). In this case, rats were perfused with 250 ml of only 2% of cold

paraformaldehyde (pH 7.4) in PBS (0.1 M). Brains were removed and kept in the fixative for approximately 1h, washed (3 x 10 min) in PBS, cryoprotected overnight at 4C in PBS containing 20% sucrose and embedded in OCT embedding medium. Consecutive frozen sections (20 μm thickness) of anterior brain and brainstem were cut and thaw-mounted onto poly-D-lysine coated slides. The sections were stained immunohistochemically according to the avidin-biotin method {6168 /id Grondahl-Hansen, Agerlin, et al. 1988}. Brain samples were blocked with endogenous peroxidase blocking buffer (3M NaN_3 and 1% H_2O_2 with 0.1% (w/v) saponin in Earls Buffered Salt Solution (EBSS)(Gibco BRL)) and Avidin/Biotin blocking kit (Vector Lab), and incubated overnight in a humidified chamber at room temperature with the primary unlabelled cytokine-specific antibody at dilution 1:50 (Goat anti-rat). Slides were then washed and incubated for 45 min with a biotinylated secondary antibody (biotinylated-donkey anti-goat IgG Fab2 diluted 1:700). After incubation, the sections were rinsed and reacted in Vectastain ABC kit (PK4002, Vector Laboratories, Burlingame, CA) for half an hour in a humidified chamber. The sections were then rinsed, treated with a solution of 3-3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-5637) and hydrogen peroxide, dehydrated and mounted in bicarbonate-buffered glycerol (pH 8.6). The control for non-specific staining of the secondary biotinylated antibody was identical to the protocol for immunofluorescent labeling described previously. Liquid pre-absorption tests were used to assess the specificity of the primary antibodies for MCP-1 and IL-

1β. The preparations were examined under a Zeiss light microscope and photographed (Kodak Tmax film). For statistical analysis, labeled cells in the brain regions of interest were counted manually and averaged over 2-4 sections per animal, and a t-test was used to compare between two unpaired averaged groups and determine significance. All counts were performed on coded slides to avoid observer bias. Comparisons between more than two unpaired groups were performed by one way ANOVA, followed by a Dunnett Multiple comparison test for post-hoc analysis. $P<0.05$ was considered statistically significant.

CHAPTER 3

NEURONAL ACTIVATION IN RESPONSE TO COLITIS

3.1 Objective # 1

Examine the CNS response to colitis by means of neuronal activation, using Fos immunohistochemistry.

3.2 Background

Studies of the CNS response to peripheral inflammation, in the endotoxin model, have illustrated two sites in the brain where neurons are activated in response to the peripheral immune challenge. The first site includes regions that are anatomically connected to the periphery, via neuronal projections, among these are the NTS, the parabrachial nucleus, and the hypothalamic paraventricular and supraoptic nuclei. The second site consists mainly of cirumventricular organs such as the OVLT, the subfornical organ, and the area postrema.

A previous study (85) has examined the effect of the acute-phase response of peripheral immune activation on the expression of the immediate early-response gene c-*fos*. A combination of immunohistochemistry and in situ hybridization was used to co-localize the expression of *c-fos* to neurons that produce the neuropeptides CRF, oxytocin (OT), or vasopressin (AVP). Immunolabeling of these neuropeptides, that are consitutively expressed in neurons of various CNS structures, was used to locate the neurons expressing *c-fos*. Intraperitoneal (i.p.) injection of lipopolysaccharide resulted in the

induction of *c-fos* mRNA expression in multiple brain regions, two of which were located in the dorsal vagal complex. These were the NTS and the area postrema.

In another study (20), the pro-inflammatory cytokine IL-1β was administered both peripherally (i.p.) and centrally (i.c.v.) to rats and resulted in a differential pattern of c-Fos expression. Although, both routes of administration of IL-1β induced c-Fos expression in circumventricular organs and activated the HPA axis, as assessed by analysis of plasma corticosterone, only peripheral administration of the cytokine induced c-Fos expression in the NTS. This suggested that peripheral immune signals could signal the brain via abdominal vagal afferent projections to the dorsal vagal complex.

Both the endotoxin-induced model of peripheral inflammation, which stimulates peripheral production of cytokines, and the peripheral administration of the potent pro-inflammatory cytokine IL-1β, lead to the activation of neurons in two specific regions within the dorsal vagal complex: the NTS and the area postrema. Colonic inflammation, which can be regarded as a peripheral immune response, is followed by a massive production of immune signals such as IL-1β. This cytokine can be produced in the inflamed colon and circulate through the blood in a way somewhat similar to the immune challenges mentioned above. Taken together, this evidence suggests that following colitis, circulating immune signals induce the expression of the early response gene c-Fos in the dorsal vagal complex.

3.3 Hypothesis

Following colitis, neurons are activated in two regions of the dorsal vagal complex, which receive peripheral neuronal and blood-borne signals, these are the NTS and the area postrema, respectively.

3.4 Methods

Three groups of adult rats (n=4; 150-200g) were intrarectally injected with TNBS and thereafter killed and perfused at two early (6,12h) and one late (72 h) time point. To assess the effects of the treatment induced stress or ethanol on neuronal activation, control groups were injected either with saline or ethanol (50%) and killed and perfused at the same respective time points. Brain sections were obtained in the same manner as previously explained. Immunohistochemistry was used for detection of the expression of early response gene c-Fos (Fos). Fos-expressing cells were counted in the regions of focus and compared to saline and ethanol treated control groups. After 48h incubation in a polyclonal rabbit anti-rat primary antibody, brain samples were washed and labeled with a fluorescent secondary antibody (donkey anti-rabbit cy3). Labeled slices were mounted on slides and observed under an immunofluorescence microscope.

3.5 Results

Both at early (6 h, 12h) and late (72 h) time points following TNBS treatment, rats developed severe colonic ulcerations, that were mild or absent in the ethanol or saline treated groups, respectively. At 72 h following the induction of colitis, the presence of colonic ulcerations was coupled to the loss of body weight, whereas both ethanol and saline control groups exhibited normal weight gain. These results are summarized in Table 3.1 (for n>2 parameters are presented as Mean ± Standard Deviation [SD]).

TABLE 3.1 The Effects of TNBS on Colonic Damage and Body Weight

Type of treatment and time (h) after administration	**Macroscopic damage score**	**Changes in body weight (g) Weight gain (+); loss (-)**
Saline 6, 12	0	-
Saline 72	0	+15.3 ± 2.16
Ethanol 12	2 ± 1	-
Ethanol 72	1	+14
TNBS 6	5 ± 2.5	-
TNBS 12	7 ± 1.4	-
TNBS 72	5.5 ± 1.3	- 14.2 ± 1.7

Table 1 reveals a high macroscopic damage score in all TNBS treated groups, that are either low or minimal (0) in the respective ethanol and saline treated groups. In addition, the TNBS treated group lost approximately 5 g per day, opposite to the ethanol and saline control groups, both of which had gained the equivalent weight (approx. 5 g/day).

Twelve hours following the induction of colitis, there was a significant increase in the expression of the early-response gene product *Fos* in the area postrema ($P<0.05$), compared to six hours post treatment, and to the saline and ethanol control groups. The amount of labeled nuclei remained elevated at 72h post TNBS treatment (Fig. 3.3). This increase was specific to the area postrema and did not occur in any other structure within the dorsal vagal complex, including the NTS. Although, there was no significant change in Fos expression in the NTS, there was a discrepancy between the number of labeled nuclei in the saline and ethanol group (Fig. 3.1;A,B). In both TNBS and ethanol treated groups, there was a large number of Fos expressing cells, in the medial and lateral portions of the NTS, that were not present in the saline control group.

To assess whether the increase in Fos expression was apparent in other circumventricular organs or in regions without any direct neuronal or blood-borne contact with the periphery, the expression of Fos was also examined in the OVLT and in the hippocampus, respectively (data not shown). In both regions, there was no change in Fos expression in the TNBS-treated compared to saline and ethanol treated groups. Due to the lack of double labeling for Fos

expression with microglial (OX-42) and astroglial (GFAP) specific markers, the cells expressing Fos are most likely neurons and not glia (Fig. 3.2).

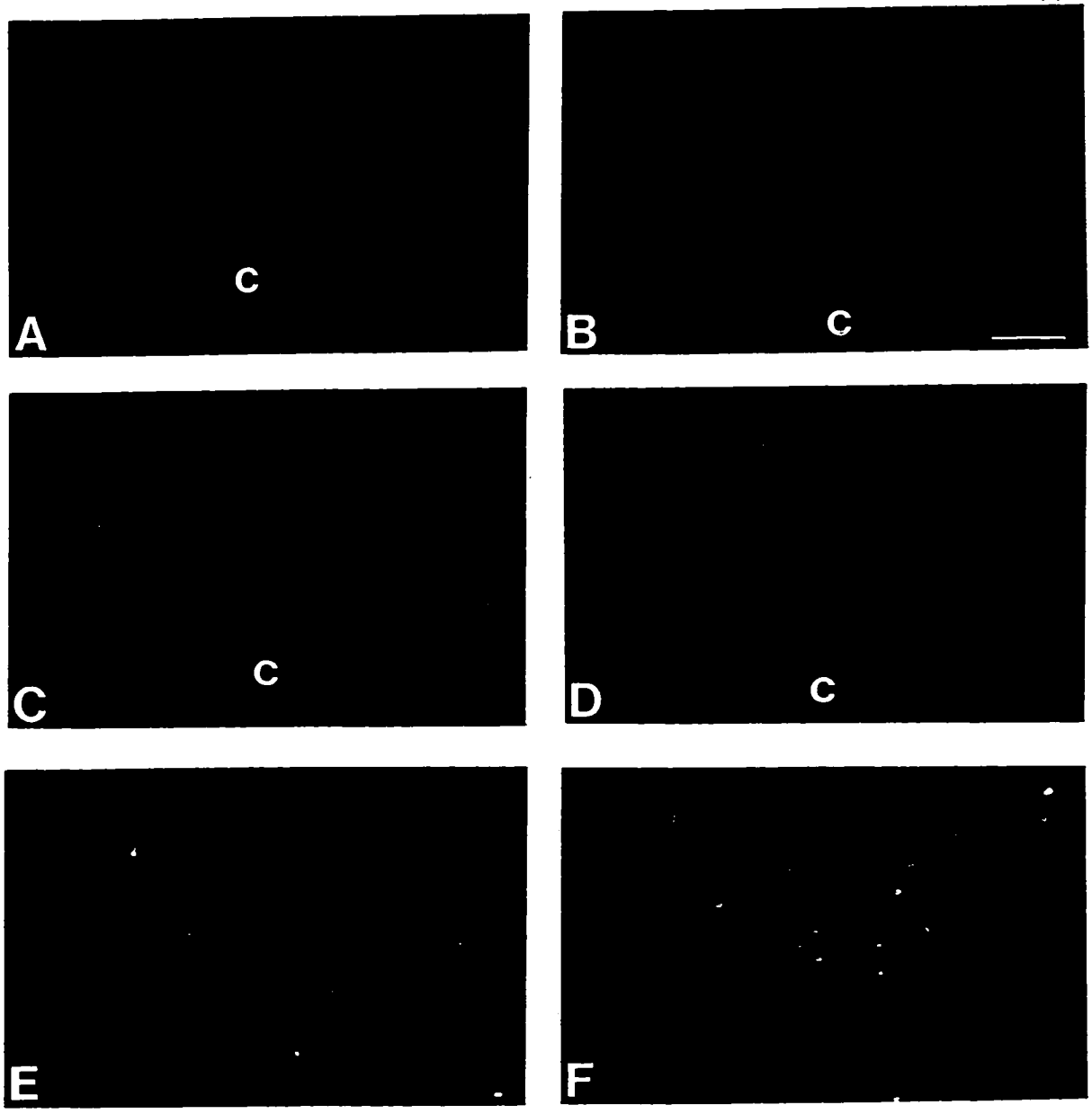

Fig. 3.1

Figure 3.1.

Fluorescence micrographs of coronal sections stained for Fos in the dorsal vagal complex from saline (A) and ethanol (B) treated animals (at 12 h after treatment) and TNBS (C, D) treated animals (at 12 and 72 h after treatment). The expression of Fos in the dorsal vagal complex was very low in the saline (A), increased and confined to the medial and lateral solitary tract in the ethanol (B), and further increased and extended to the area postrema at both early (C) and late (D) time points in the TNBS treated animals. A higher magnification of Fos expression in the area postrema (E) and nucleus of the solitary tract (F) in the TNBS treated animals (72 h) is displayed in the two bottom micrographs. c, central canal. Scale bar: A-D=100μm; E,F=50μm.

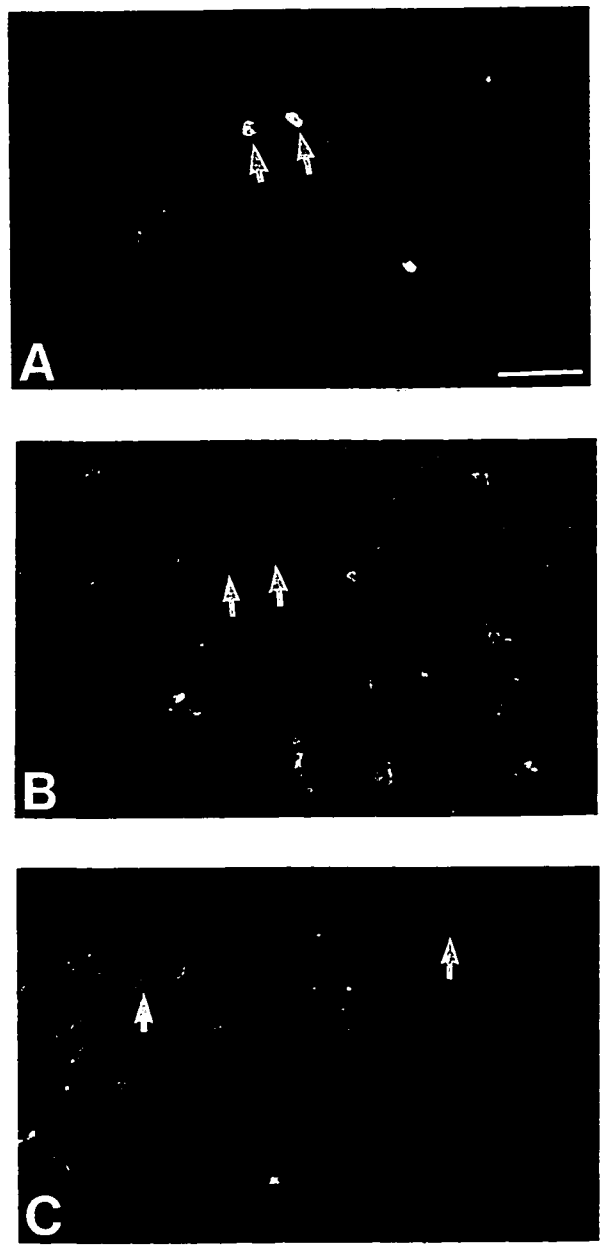

Fig. 3.2

Figure 3.2.

Fluorescent micrographs of double-labeled non-overlapping coronal sections stained for Fos and either microglia or astrocytes. Fos immunoreactive nuclei (A,C) do not overlap with microglia (OX-42)(B) and astrocytes (GFAP)(C). In (A,B) arrows point at two sites of non-overlapping staining for Fos and microglia and in (C) the right arrow points to a Fos labeled nuclei whereas the left arrow is facing an astrocyte. Scale bar= 25μm.

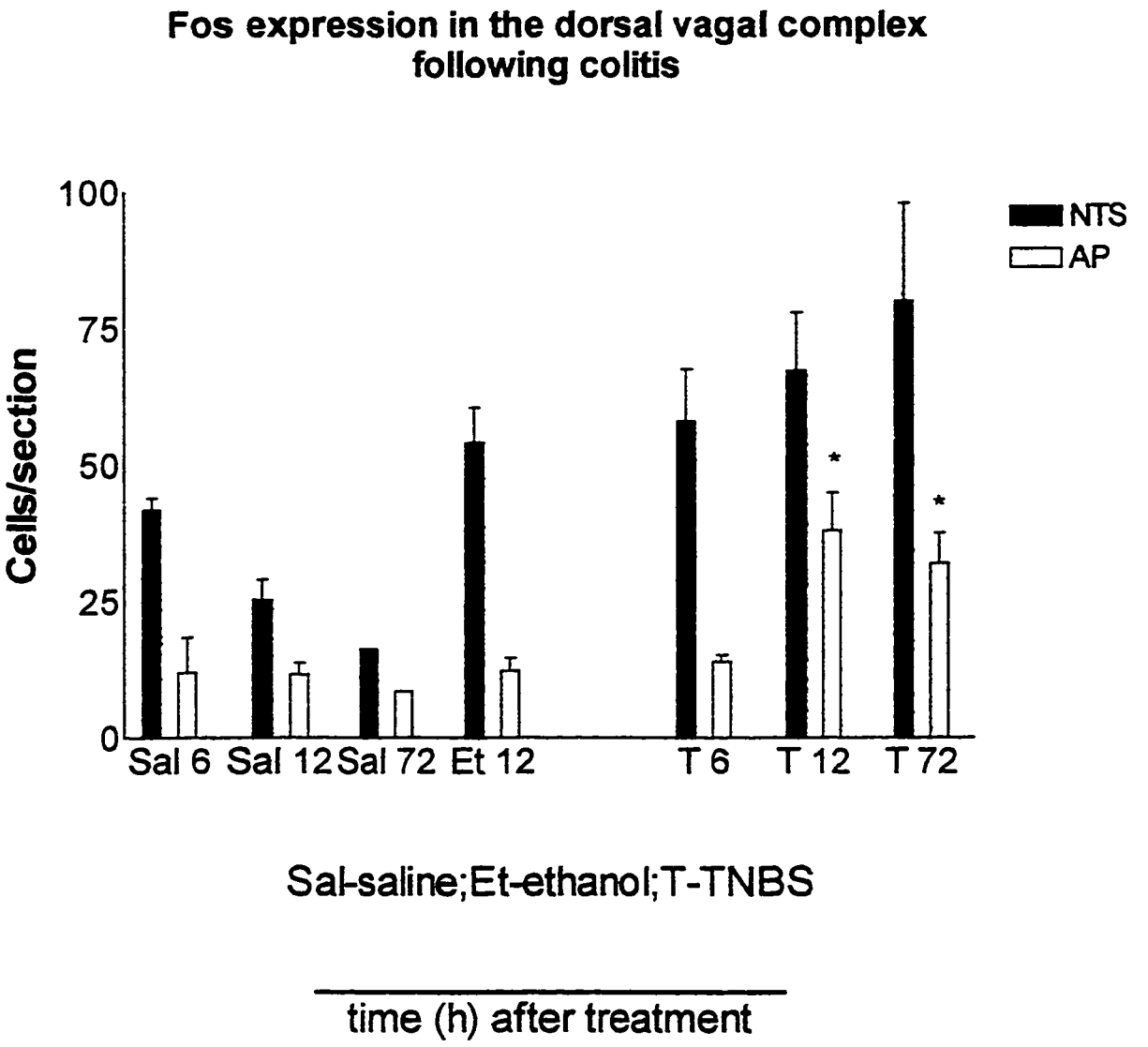

Figure 3.3. Fos expression in the dorsal vagal complex following colitis. There were no significant differences between the ethanol and TNBS group in the NTS at 12h, though both were significantly greater than saline. All control groups were significantly lower than TNBS groups at 12h and 72h in the AP (* $P<0.05$). P values were calculated by one-way ANOVA and compared by multiple post tests comparisons (Dunnett). Bars represent mean ± SD.

3.6 Discussion

We have demonstrated the expression of Fos in the area postrema, at 12 h and 72 h after the induction of colitis. Due to previous literature demonstrating the expression of this early response gene as a specific marker of neuronal activation and the lack of double-labeling of Fos with either microglial (OX-42) or astrocytic (GFAP) specific primary antibody, we conclude that Fos expressing cells are probably neurons. The increase in Fos expression was specific to the area postrema and did not occur in any other structure within the dorsal vagal complex. In addition to the area postrema, another circumventricular organ, the OVLT, was examined and had no significant change in Fos expression in response to colitis. For this reason, we have postulated that the increase in the expression of Fos in the area postrema might not be entirely attributed to the lack of an intact blood-brain barrier, which allows the free passage of potential neuronal activators, and is likely to depend on other intrinsic factors in the dorsal vagal complex.

Although, both ethanol and saline control groups had no effect on Fos expression in the area postrema, the ethanol treated group exhibited a marked increase in the expression of Fos in the medial and lateral portions of the NTS. This suggested that ethanol treatment alone, activated neurons projecting to the NTS, excluding the area postrema. The neurons activated within the area

postrema were probably unaffected by the ethanol and might have been induced to express Fos either directly or by other factors either produced in or derived from the inflamed colon.

The effect of ethanol on neuronal activation in the NTS is in line with another study (74) demonstrating the evoked neuronal activation in the paraventricular nucleus of the hypothalamus by peripheral administration of alcohol. In this study, the intraperitoneal (but not intragastric) administration of alcohol induced the expression of Fos and two other ACTH secretagogues, CRF and AVP. Moreover, this finding has linked the full expression of *c-fos* mRNA and Fos protein to the activation of the hypothalamic-pituitary-adrenal axis. Hence, peripheral administration of ethanol alone can induce the activation of the HPA axis, which receives neuronal afferent input from the periphery via the NTS (56). In the same fashion, the intrarectal administration of either TNBS in ethanol or ethanol alone lead to the induction of Fos expression in the NTS, indicating that the effects of ethanol, administered by this route, on neuronal activation, could mimic some of the effects of intraperitoneal administration of this drug. However, the expression of Fos in the area postrema was not induced by ethanol alone, and only appeared in the TNBS (in ethanol) treated group. This suggests that neurons within the area postrema are less sensitive to activation by ethanol than their counterparts in the NTS, and their activation could be induced by blood-borne cytokines or other circulating products of the inflamed colon. These neurons have been shown to express receptors for

peripheral immune signals, such as IL-1β or IL-6 (24). However, in our study, the expression of c-fos in response to colitis in other circumventricular organs that express these receptors, such as the OVLT, remained unchanged before and after TNBS treatment. Therefore, the activation of neurons in the area postrema could not be solely ascribed to the availability of neuronal receptors for these cytokines. We suggest that the combined effects of neurons activated in the NTS, which project to the area postrema, and cytokines originating from the inflamed colon lead to the activation of neurons in the area postrema.

Our finding links the model of hapten-induced colitis to a brainstem region which is known to be involved in the IL-1β-induced activation of the HPA-axis (45). This is the area postrema. Although, neurons in the NTS were probably induced to express Fos by ethanol, neuronal activation in the area postrema correlated with the extent of macroscopic colonic damage and the colonic expression of the pro-inflammatory cytokine IL-1β (data not shown). The activation of neurons in the area postrema that project to the paraventricular hypothalamic nucleus leads to hypothalamic secretion of norepinephrine, however, whether this event, which is associated with the activation of the HPA axis, is a feature of colitis remains to be determined.

CHAPTER 4

MICROGLIAL ACTIVATION IN RESPONSE TO COLITIS

4.1 Objective #2:

Demonstrate the activation of microglia in response to colitis

4.2 Background

Peripheral immune signals, which accompany inflammation, do not exclusively affect neurons. Microglia, the brain's resident immune cells, can also be activated in the brains of immune challenged rats. The activation of microglia could be induced by peripheral administration of either the immune stimulator lipopolysaccharide (16) or by the proinflammatory cytokine IFN-γ (103) (108), and occurs preferentially in brain parenchyma and in the vicinity of circumventricular organs (16).

Following activation, a number of changes occur in microglia: a morphological transition from the ramified resting to a round/ameboid shape, the induction of major histocompatibility complex class II antigen, and in some cases the transcription (15) and production (101) of IL-1β. Due to the low levels of MHC class II expression in resting microglia and the high expression of this antigen in activated microglia (at late time points), it is commonly used as a marker for the late activation of microglia.

In brains of normal rats, most microglial cells are resting and express very little MHC class II antigen. Expression of MHC class II is confined to circumventricular organs, and to brain vasculature and parenchyma. Crohn's colitis involves the production of the pro-inflammatory cytokines (IL-1β and IFN-

γ) whose peripheral administration has been shown to induce microglial activation (108). The capacity of IFN-γ to induce microglial activation and expression of MHC class II antigen was shown both *in vitro* (103) and *in vivo* (108). This suggested that circumventricular organs, such as the area postrema and the OVLT, could be sites where resident microglia are exposed to potential blood-borne stimulators. Hence, we hypothesized that colitis is followed by the peripheral production of cytokines such as IFN-γ that induce the activation of microglia and expression of MHC class II antigen (108).

4.3 Hypothesis

Microglia are activated in response to colitis.

4.4 Methods

To assess microglial activation following the induction of colitis, various groups of adult rats (n=4-6; 150-200g) were given intrarectal injection of TNBS, and subsequently killed and perfused at varying time points (6,12, and 72 h). Perfused brains were kept in fixative and cryostat sectioned to a thickness of 30 μm.

To label microglia, brain slices were then stained with a primary antibody specific for macrophage/microglia marker complement receptor-3 ((CR-3); OX-42). Microglial activation was labeled with another primary antibody specific for

antigen presenting cell marker MHC class II antigen (OX-6). As astrocytes can also express MHC class II, samples were double labeled with MHC class II (OX-6) and astrocyte (GFAP) specific antibodies. To control for the systemic effect of TNBS on microglial activation, an additional group (n=2) was intravenously injected (i.v.) with TNBS dissolved in water. All groups were compared to saline and ethanol controls.

4.5 Results

Seventy-two h after TNBS treatment, severe ulcerations were present in the colons of rats (average damage score 5.5 ± 1.3 SD). Control groups treated with ethanol or saline had mild or no apparent ulcerations (average damage scores 1 and 0, respectively). Colitis was followed by the loss of body weight (average loss ~5g per day), opposite to the normal daily weight gain (~ 5g) observed in the ethanol and saline treated groups. Histological and macroscopic damage scores correlated well as the TNBS treated group had a higher score (Histology score 6.3 ± 2.75 SD) than the saline control group (0.83 ± 1).

Three days following the induction of colitis, there was an up-regulation and induction of MHC class II expression in the area postrema (Fig. 4.3) accompanied by a change in microglial morphology and an increased staining intensity with the macrophage/microglia specific primary antibody (OX-42)(Fig. 4.1, 4.2A). The expression of MHC Class II was confined to the area postrema

and did not appear in any other dorsal vagal structure. Saline and ethanol control groups expressed very little MHC class II antigen, at all time points examined (6h, 12h, 24h, and 72h), and high levels of microglial specific marker complement receptor-3 (OX-42) (Fig. 4.1 F). In control groups, cells labeled with the latter primary antibody had a ramified shape suggesting that they were resting microglia. In another circumventricular organ, the OVLT, there was no change in MHC class II antigen expression, following TNBS treatment. Although, intravenous administration of TNBS appeared to have no effect on the expression of class II MHC antigen in the dorsal vagal complex or in the OVLT, at the time point studied, it was followed by an increase of MHC class II expression in the choroid plexus (not shown). There was a lack of double-labeling with MHC class II (OX-6) and astrocyte (GFAP) specific primary antibodies in all examined regions (Fig. 4.2 B,C), however, a few cells appeared to coincide in the vicinity of brain vasculature (central canal, lateral ventricles, and OVLT).

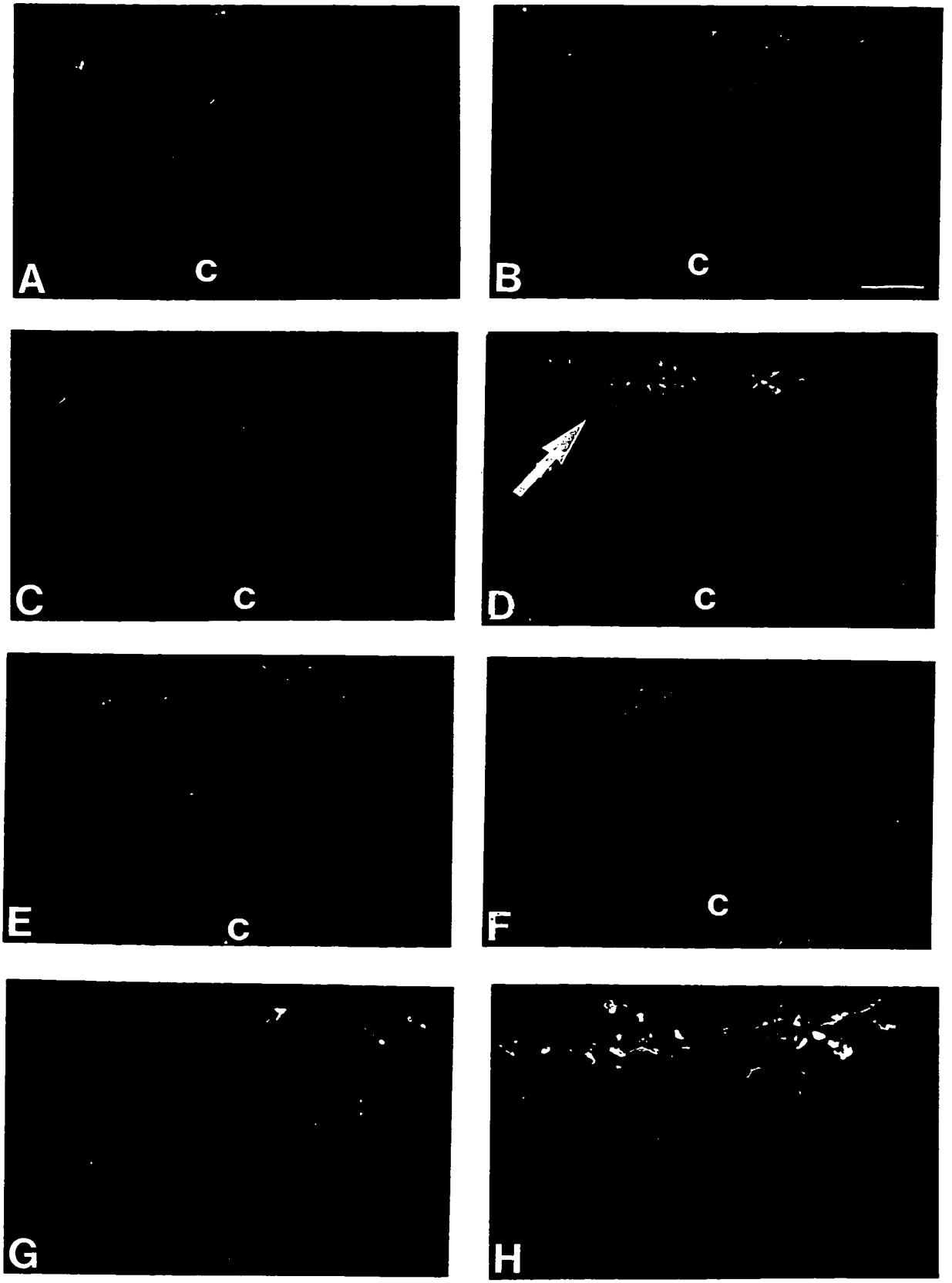

Fig. 4.1

Figure 4.1.

Fluorescence micrographs of coronal sections stained for MHC class II (OX-6) and microglial (OX-42) specific markers in the dorsal vagal complex from saline (A), ethanol (B), TNBS (i.v.) (C), TNBS (rectal) (D), and TNBS + dexamethasone (E) treated animals. A higher magnification of MHC class II expression is shown from ethanol (G) and TNBS (H) treated animals. Sections stained for microglial specific marker (OX-42) are shown in (F). All micrographs were taken from animals killed at 72 h following treatment. c, central canal. Scale bar: A-F=100μm; G,H=50μm.

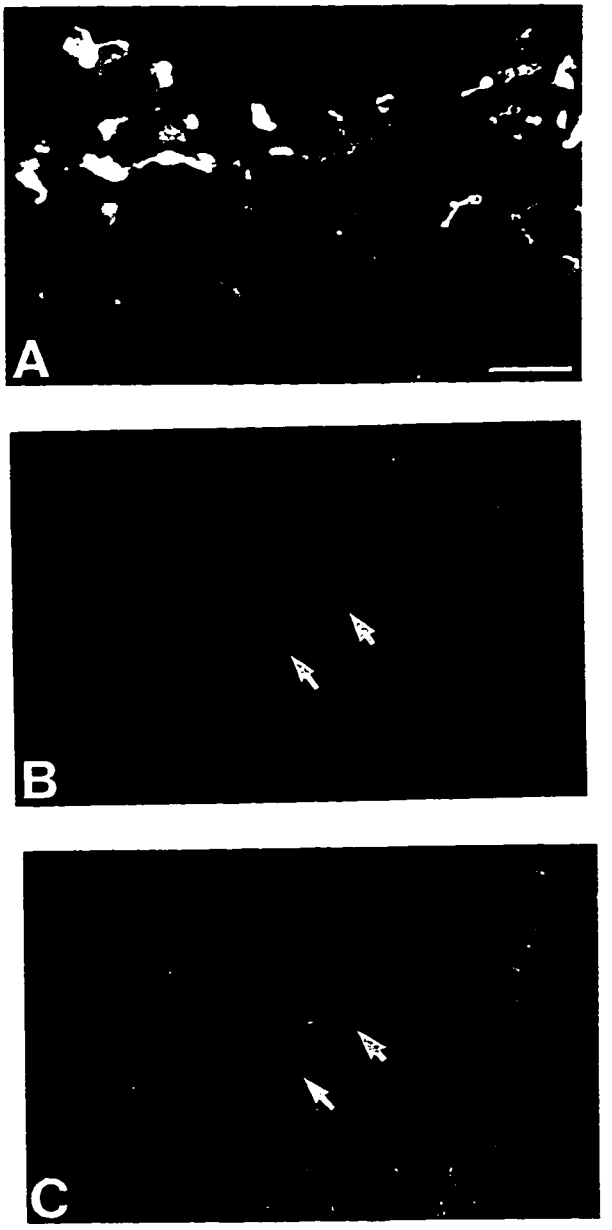

Fig. 4.2

Figure 4.2. Fluorescence micrographs of showing a high magnification of coronal sections stained for immunoreactive MHC class II expression (A) and the lack of overlap between MHC expressing cells and astrocytes (B,C). Arrows in (B) and (C) point at two MHC class II expressing cells (OX-6)(B) which do not coincide with astrocytes (GFAP)(C). Scale bar: A-C=25μm.

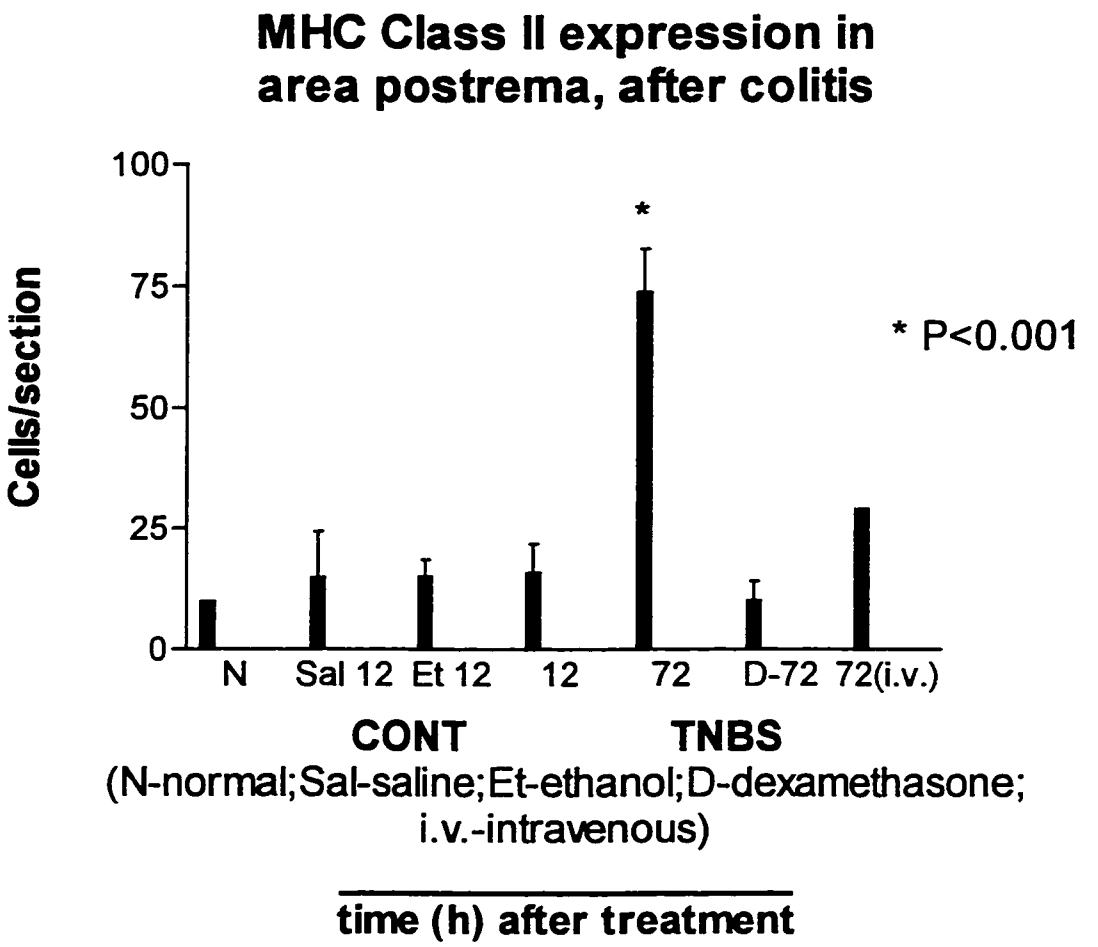

Figure 4.3. MHC class II expression in the area postrema after colitis. * P<0.001 was obtained (One way ANOVA) for the saline (12 h), ethanol (12 h), and dexamethasone pretreated (72 h) control animals. For n>2 bars represent Mean ± SD.

4.6 The appearance of activated microglia in the area postrema of interleukin-10 knockout mice.

4.6.1 Background

Additional support for the hypothesis that microglia are activated in response to colitis was to use another experimental model of inflammatory bowel disease, this is the IL-10 knockout mice. In this model, mice, generated by gene targeting and deficient in the anti-inflammatory cytokine IL-10, develop spontaneous chronic enterocolitis (54). The role of IL-10 in brain-gut interactions will be explained first how it regulates immune responses and gut inflammation and then its function in the central nervous system.

Interleukin-10 is an anti-inflammatory cytokine produced by T cells, B cells, or monocytes activated by endotoxin (44). It suppresses the production of cytokines by activated T lymphocytes (Th 1) under conditions requiring the presence of antigen presenting cells. IL-10 can significantly reduce antigen-specific T-cell proliferation by diminishing the antigen presenting capacity of monocytes via down-regulation of the expression of class II major histocompatibility antigen (42). Moreover, this cytokine inhibits the production of cytokines by activated macrophages or monocytes (e.g., endotoxin-induced production of IL-1β, 6, or tumor necrosis factor-α) (12;75). Hence, it is involved both in the regulation of T cell activation and in the down-regulation of acute inflammatory responses.

The anti-inflammatory properties of IL-10 extend to the bowel. The role of IL-10 in the immune homeostatic regulation of the gut has been demonstrated by the generation of IL-10 deficient mice, which develop chronic enterocolitis that can be blocked by the administration of IL-10 (83). This cytokine was shown to down-regulate the activation of bowel-derived mononuclear phagocytes (*in vitro*).

In the central nervous system, the functions of IL-10 are less clear. Various *in vitro* studies demonstrate the capacity of IL-10 to inhibit microglial production of cytokines. For example, it suppresses microglial production of the pro-inflammatory cytokine TNF-α (61) and IL-12 (1). In addition, IL-10 can down-regulate the expression of molecules involved in the presentation of antigens, such as MHC class II (21) and the co-stimulatory molecules B7-1 and B7-2 (70). Hence, IL-10 is a potent anti-inflammatory cytokine capable of 'turning off' microglial production of cytokines and antigen presentation.

One can speculate on the possible scenario in the gut as well as in the CNS, in the absence of IL-10. In the gut, the lack of IL-10 production could lead to prolonged and excessive spontaneous expression of pro-inflammatory cytokines, as seen in the colons of IL-10 deficient mice. On the other hand, the lack of this cytokine in the milieu of the CNS could render microglia more sensitive to opportunistic activation. These potential conditions have led us to hypothesize that, similar to the findings in TNBS treated rats, IL-10 deficient mice are characterized by an increase in microglial activation in the area postrema.

4.6.2 Extended Hypothesis

Mice, deficient of gene encoding for the anti-inflammatory cytokine IL-10, are characterized by an increased number of activated microglia in the area postrema.

4.6.3 Methods

One group of 129/SvEv IL-10 -/- mice (n=4; 20-25g) were generated (kindly provided by Dr. Paul Kubes, University of Calgary) and used together with a wild type control group (n=2; 20-25g) (5). All manipulations were performed in much the same way as previously described. To assess the severity of enterocolitis, colons were removed, damage scored, and samples removed for histological examination and MPO activity measurement as previously described. Mice were then perfused, with saline and cold paraformaldehyde (4%), by using a 10ml syringe and inserting a 26 gauge needle into the left ventricle. Brains were then removed, incubated overnight in the same fixative, and transferred to a sucrose solution (20% in PBS) for another overnight incubation.

Frozen sections were then cut, at the same levels as described elsewhere, to a thickness of 30μm, and the brain regions of interest examined. Immunohistochemistry was used for detection of microglia (OX-42) and the expression of MHC class II antigen (OX-6). Both microglial (OX-42) and MHC class II (OX-6) specific primary antibodies cross-reacted with the mice form of

these markers, and generated a staining intensity and cellular morphology similar to the one apparent in rat-derived brain samples.

4.6.4 Results

There was an increase in the number of cells expressing MHC class II antigen in the area postrema of mice deficient for the gene encoding IL-10. A close examination of the morphology of cells expressing MHC class II revealed multiple structures characteristic of microglia in varying stages of activation (Fig. 4.4). These include both the ramified and the round/ameboid shapes. The quantification of MHC class II appeared somewhat variable, and thus the increase in MHC class II in the IL-10 deficient mice compared to the control group was not significant (Fig. 4.5). However, it should be noted that the mice used in this study were at a stage three weeks prior to the appearance of severe enterocolitis, hence the abdominal production of pro-inflammatory cytokines might have been insufficient to induce a significant change.

The wild type control group had a different staining distribution than the corresponding rat control groups, which could be attributed to differences between species. Unlike the non-inflamed rats, the mice control group expressed MHC class II antigen along the border of the area postrema and the NTS and close to the central canal. Moreover, the meninges dorsal to the area postrema and lining the forth ventricle of the mice control group had a lower level of MHC class II expression than its rat counterpart.

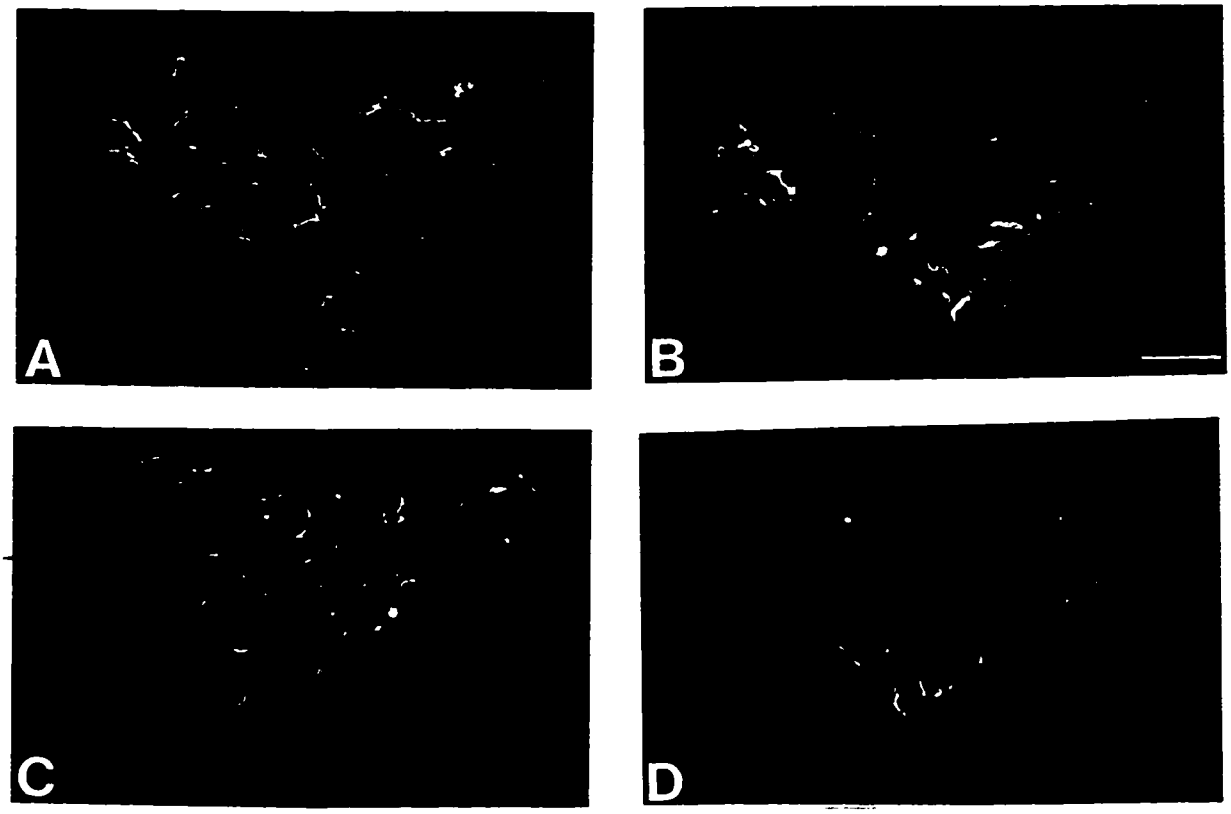

Fig. 4.4

Figure 4.4.

Fluorescence micrographs of coronal sections stained for MHC class II (OX-6) (C,D) and microglial (OX-42) (A,B) specific markers in the dorsal vagal complex from wild type (B,D) and IL-10 -/- mice(A,C). Scale bar: A-D=50μm

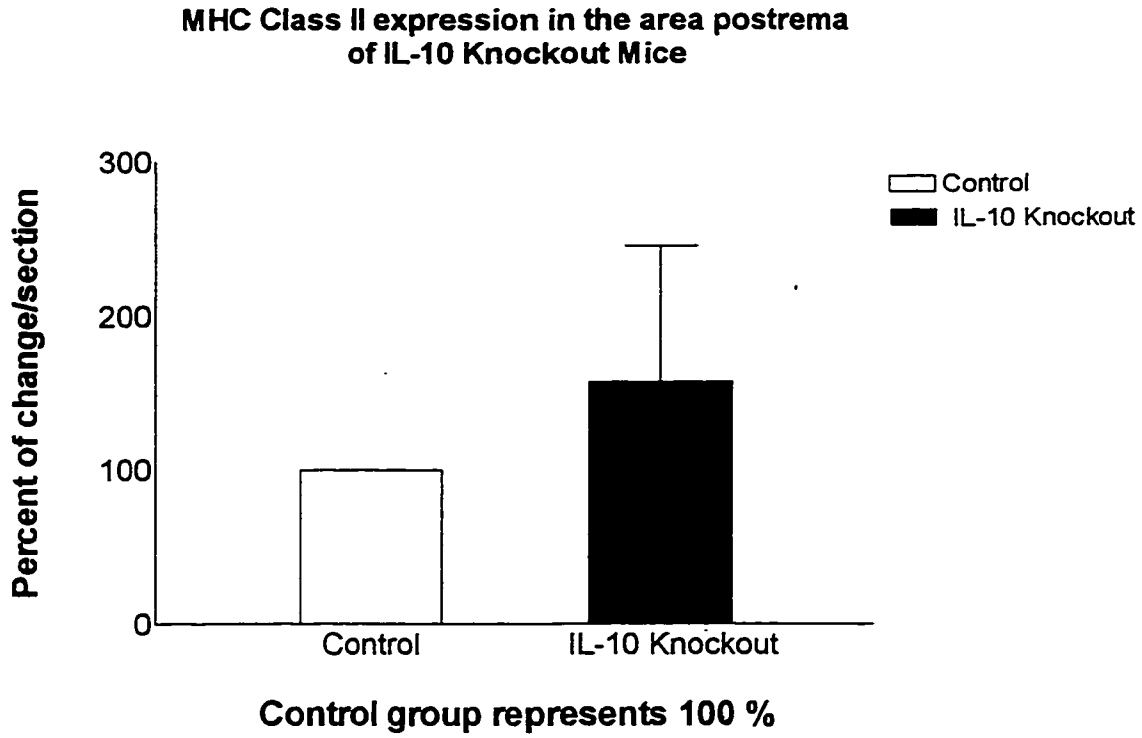

Figure 4.5. MHC class II expression in the area postrema of IL-10 knockout mice. Bars represent the percent of the control group (100%). N=2 for the wild type and 4 for the IL-10 -/- groups.

4.7 Discussion

We demonstrated the induction of MHC class II expression in the area postrema in response to hapten-induced colitis, at 72 h following treatment. Due to the morphology of cells labeled with MHC class II specific antibody, the labeling of adjacent sections with microglial specific antibody (OX-42), and the lack of double labeling with astrocytic (GFAP) and MHC class II (OX-6) specific primary antibodies, we suggest that the cells expressing MHC class II antigen are microglia. As other brain regions, such as the circumventricular organ the OVLT, were examined and did not show a significant change in MHC class II antigen expression in response to colitis, we propose that this phenomenon is specific to the area postrema. The examination of the ethanol treated group revealed no change in the extent of MHC class II expression both at early (12 h) and late (72 h) time points, and had normal macroscopic damage score, MPO levels, and colonic IL-1β immunoreactivity (not shown). This suggested that ethanol-mediated neuronal activation in the NTS is not sufficient to activate microglia in the adjacent area postrema. Yet, the induction of the marker for neuronal activation Fos (12 h after colitis) particularly in the area postrema prior and parallel to the appearance of activated microglia (72 h after colitis) within the same region, implies that these events could be related.

The intravenous administration of TNBS alone (in saline) failed to induce the expression of MHC class II in the area postrema, although, it was followed by an increased MHC class II expression in the meninges and choroid plexus. From

this observation we postulated that the activation of microglia is less likely to be mediated directly by the hapten trinitrobenzenesulfonic acid. Furthermore, pretreatment with the anti-inflammatory glucocorticoid dexamethasone (2mg/kg) abolished the increase in MHC class II expression in response to colitis and correlated with a reduction in colonic macroscopic damage score, MPO levels, and expression of immunoreactive IL-1β. These findings indicated that microglial activation in response to colitis depended on and correlated with the extent of colonic inflammation and cytokine production. The ability of dexamethsone to inhibit microglial and peritoneal macrophage activation in response to IFN-γ (21;64) suggests that microglial activation in response to colitis could be mediated by this cytokine.

We further show that the increase in microglial expression of MHC class II in response to colitis is not restricted to the hapten model of Crohn's disease, and extends to the model of enterocolitis in IL-10 deficient mice. In these preliminary observations, we have examined mice deficient of the gene encoding for the anti-inflammatory cytokine IL-10 two to three weeks before they develop severe chronic enterocolitis and observed a change in the number of MHC class II expressing cells in the area postrema compared to controls. Although, the increase in the number of cells that express MHC class II antigen was not statistically significant we argue that the mice examined had a mild level of colonic damage, which would increase at a later stage. However, the expression of MHC class II in IL-10 deficient mice at a later stage of severe colonic

inflammation remains to be investigated.

In summary, we have shown an up-regulation and induction of the marker for microglial activation MHC class II antigen in the area postrema in response to colitis, at 72 h after treatment. Intravenous administration of the hapten or pretreatment with dexamethasone did not result in a similar observation, suggesting that microglial activation was not directly mediated by the hapten alone and was more likely a feature of the extent of colonic inflammation and cytokine expression. One possible mechanism of microglial activation could be mediated by interacting with their potent stimulator IFN-γ, however, it fails to explain why the increase in MHC class II antigen presentation was restricted to the area postrema and did not occur elsewhere in the dorsal vagal complex or circumventricular organs. Hence, we have postulated that genuine features specific to the area postrema contributed to this phenomenon. The most prominent feature of the area postrema, which does not characterize other circumventricular organs, is the proximity to peripheral signals derived from both neuronal (via the NTS) and blood-borne (via the fourth ventricle) pathways.

CHAPTER 5

ROLE OF THE VAGUS NERVE IN MEDIATING MICROGLIAL ACTIVATION IN RESPONSE TO COLITIS

5.1 Objective #3

Establish whether microglial activation in response to colitis is mediated by a neural route of communication or by a blood-borne route.

5.2 Background

The *in vivo* mechanisms involved in regulating microglial activation are currently unclear. Most proposals aimed at explaining how peripheral administration of endotoxin or the cytokine IFN-γ activates microglia have concentrated on blood-borne routes of communication. This came from the observation that following peripheral immune stimulation, microglia were activated preferentially in circumventricular organs. In these CNS structures, microglia are exposed to cytokines, which are produced and released in the immune-challenged periphery. However, many signals that have the capacity to activate microglia reach the CNS via neuronal projections. For example, the central production of the cytokine IL-1β, which often occurs in activated microglia (102), has been shown to be mediated by the vagus nerve, following peripheral administration of either lipopolysaccharide (58) or IL-1β (39). In our observations, neurons and subsequently microglia were activated in the area postrema, a region with extensive vagal projections. These include both afferent projections that carry abdominal sensory input to the NTS as well as efferent projections from this nucleus to the dorsal motor nucleus of the vagus. Hence, we have hypothesized that microglial activation, following colitis, is mediated by

the vagus nerve.

5.3 Hypothesis

Microglial activation, in response to colitis, is mediated by the vagus nerve.

5.4 Methods

To test the hypothesis that the vagus nerve is involved in mediating microglial activation following colitis, a procedure was performed by which both the anterior and posterior vagal branches on the esophagus were sectioned. This procedure, also called subdiaphragmatic vagotomy, ensures the complete blockade of both afferent and efferent projections between the abdomen and the CNS, while retaining vagal innervation of the heart and lungs. Two groups of male Wistar rats (n=5-7; 150-180g) were vagotomized, allowed recovery for 10 days, and thereafter intrarectally injected with either TNBS or saline. Three days following either treatment, which is the time point of maximal activation, animals were killed and perfused. The assessment of vagal integrity was carried out by injecting (i.p.) and observing the uptake of the fluorescent neuronal tracer Fast blue. Fluorescence microscopy was used to determine the presence of Fast blue in the area postrema, dorsal motor nucleus of the vagus (DMN), and nodose ganglia. Fluorescence in the area postrema was an indicator of successful

uptake of the tracer, afferent denervation was verified by examination of fluorescently labeled cells in the nodose ganglia and vagal efferent integrity assessed following examination of labeled cells in the DMN. All other methods for labeling and determining the activation of microglia were identical to those described in the previous chapter.

5.5 Results

The macroscopic examination of colons of vagotomized rats revealed moderate ulcerations (damage score 4 ± 1.92 SD), that were absent from the respective saline treated group (damage score 0). Both saline and TNBS treated vagotomized groups gained weight following either treatment (the TNBS and saline treated groups gained each approximately 5.1 and 4 g per day). The MPO levels of the TNBS treated vagotomized group (MPO levels 23.4 ± 33.4 expressed in mUnits/mg/min) were lower (not significantly) than in the TNBS treated non-vagotomized group (MPO levels 48.0 ± 65.4). The saline treated vagotomized group had low levels of MPO (MPO levels 6.8 ± 1.4). The histological examination of the colons of TNBS treated vagotomized rats revealed a significantly higher score (5 ± 2.6 SD) than in the respective saline treated group that was comparable to the non-vagotomized TNBS treated group (6.3 ± 2.75 SD).

Vagotomy attenuated the increase in the expression of MHC Class II antigen, apparent at 72h following the induction of colitis (Fig. 5.1). Both TNBS

and saline treated groups expressed low amounts of this antigen similar to the non-vagotomized saline and dexamethasone pretreated groups (Fig. 5.2). This observation is specific to the area postrema and refers to cells that were labeled with MHC Class II (OX-6) and microglia/macrophage (OX-42) specific antibodies. Other circumventricular organs, for example the OVLT, were not affected by the operating procedure. However, in two other anatomical structures, which are related to the vagus nerve, there is a significant increase in MHC Class II antigen expression, these are the NTS and the dorsal motor nucleus of the vagus. In all of the vagotomized groups, staining with the MHC Class II specific antibody revealed a mix of round/ameboid and ramified cells, with microglial morphology, in both nuclei which normally are devoid of the expression of this antigen. This is in line with a previous report (84) that demonstrated the presence of activated microglia in these brain nuclei fourteen days following cervical vagotomy. All vagotomies were verified and proven to be successful as seen by the examination of the uptake of the neuroanatomical retrograde tracer Fast blue. The area postrema was stained positively, indicating a vascular uptake, whereas the dorsal motor nucleus of the vagus and the NTS were virtually devoid of Fast blue labeled neurons (Fig. 5.1 F).

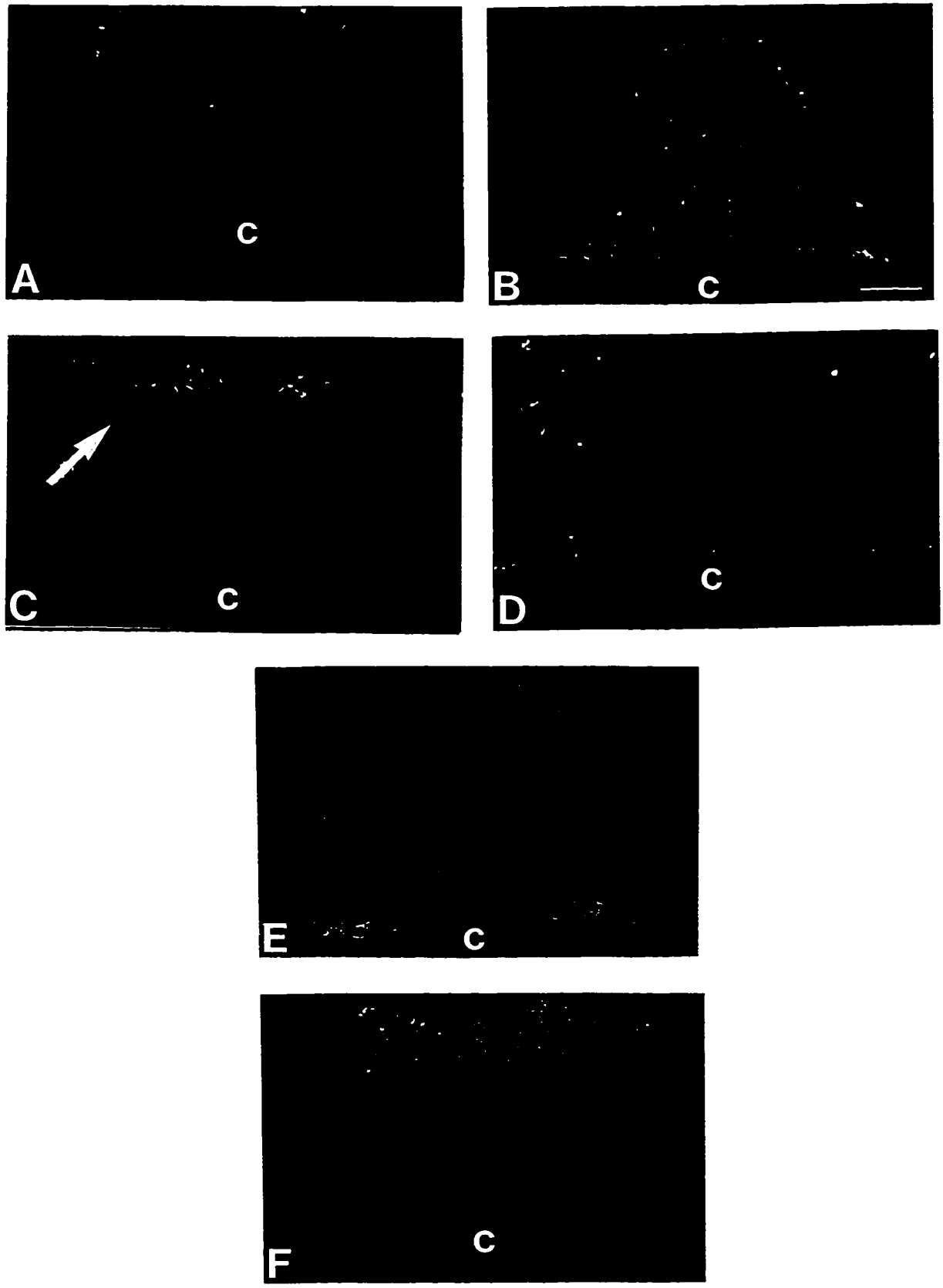

Fig. 5.1

Figure 5.1.

Fluorescence micrographs of coronal sections stained for MHC class II (OX-6) (A-D) and microglial specific markers (OX-42) in the dorsal vagal complex from saline (A,B) and TNBS (C,D) treated animals (at 72 h after treatment). The expression of MHC class II was low in the non-vagotomized saline (A) and vagotomized saline (B) and TNBS (D) treated animals, and was high in the non-vagotomized TNBS treated animals. Both vagotomized saline and TNBS treated animals expressed MHC class II in the nucleus of the solitary tract and the dorsal motor nucleus of the vagus. The staining for microglial specific marker (OX-42) in the control animal is shown in the bottom micrograph (E). The presence of the neuronal tracer Fast Blue was specific to the area postrema and was not detected in the dorsal motor nucleus of the vagus and the NTS of vagotomized rats (F). Arrow in C points at cells on the border of the AP and NTS. c, central canal. Scale bar: A-E=100μm.

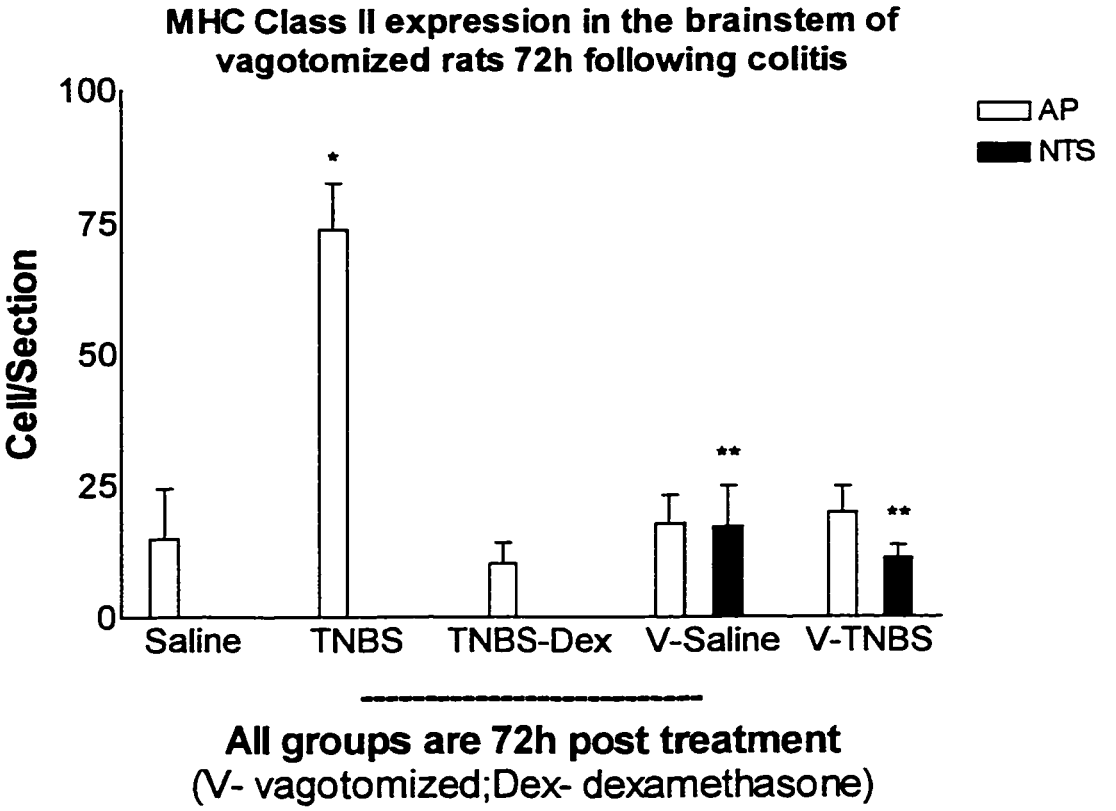

Figure 5.2. MHC class II expression in the dorsal vagal complex of vagotomized rats at 72 h following colitis. In the AP there was a significant reduction in MHC Class II expression compared to the TNBS group (* $P<0.05$) and the levels were similar to saline-treated, non-vagotomized rats. There was also a significant increase (from 0) in the NTS after vagotomy (Single group t- test; saline vs saline vagotomized; TNBS vs TNBS vagotomized; ** $P<0.05$). All bars are represented as Mean ± SD.

5.6 Discussion

This study revealed for the first time a vagally mediated pathway of microglial activation in response to peripheral inflammation. Following vagotomy, the induction of colitis by intrarectal injection of the hapten trinitrobenzenesulfonic acid (TNBS), which normally leads to the activation of microglia in the area postrema, had no effect on the extent of microglial expression of MHC class II antigen in that region. Vagotomy did not significantly reduce the severity of colitis as assessed both macroscopically (damage score) and microscopically (MPO levels). This finding puts in focus the role of abdominal vagal branches in carrying peripheral signals to the CNS, that subsequently lead to the activation of microglia. It further suggests that the pathway of microglial activation could not be entirely ascribed to blood-borne signals, such as IFN-γ, and might also involve centrally mediated signals derived from the intact vagus nerve. One possible signal might be the neuropeptide that is known to be produced by neurons in the NTS, substance P (66). This neuropeptide has been shown to activate microglia (*in vitro*), and induce the production of the cytokine IL-1β, although not via the common NK-1 receptor (67). Another conceivable way by which microglia could be activated is via purinergic receptor, as they express functional P2Z purinoreceptors (27). The source of extracellular ATP could be NTS neurons that regulate cardiovascular and respiratory functions via this neurotransmitter (80).

Our additional observation, of the appearance of MHC class II expressing

activated microglia, in the NTS and the dorsal motor nucleus of the vagus, is consistent with previous literature (84). Microglia are known to respond to neuronal degeneration, that accompanies vagotomy, by proliferating around the lesioned neurons, and removing the synaptic terminals from the neuronal surface (96). Our data is in line with the previous work of Rinaman *et al.* (84) in that it reveals the persistent presence of activated microglia at the site of neuronal degeneration as late as two weeks after the lesion. However, a very recent study (73) may shed some light and provide insights for a possible explanation for this phenomenon. In this study, the authors investigated the conditions that maintain low expression of microglial MHC class II expression in the normal rat brain. Microglia in mixed explant cultures of neonatal rat hippocampus, that were exposed to the cytokine IFN-γ, expressed very low levels of MHC class II, but were induced to express much higher levels of this antigen after blockade of neuronal activity by neurotoxins (tetrodotoxin (TTX), ω-conotoxin) or glutamate antagonists. The inducibility of MHC class II was enhanced by neutralizing the neurotrophins produced locally within cultures and was inhibited by the addition of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), or neurotrophin-3 (NT3). In addition, both NGF and to a lesser extent NT3 acted directly on the microglial p75 neurotrophin receptor as demonstrated by blockade of this receptor with antibodies. This finding clearly indicates that electrically active neurons can continuously secrete neurotrophins capable of maintaining a basal low level of MHC class II

expression in the CNS. In light of another recent study showing the accumulation of these neurotrophins in vagal afferent neurons (41), we suggest that vagotomy leads to a decrease in the expression and secretion of neurotrophins that normally inhibit MHC class II expression in the NTS and the dorsal motor nucleus of the vagus. The reduction in the extracellular levels of neurotrophins might have primed resident microglial cells to activation by potential stimulators diffusing through the area postrema to these nuclei or originating from degenerating neighboring neurons.

The vagus nerve could mediate the signals leading to microglial activation in the area postrema, by normally maintaining low levels of MHC class II expression via active neuronal secretion of neurotrophins from the NTS into the area postrema. However, peripheral immune challenge could result in the production of cytokines that signal abdominal vagal afferent nerves to reduce the extent of neurotrophin secretion. This, in turn, might lead to the removal of the mechanism presented above, which suppresses antigen presentation, resulting in an increase in MHC class II expression. As this scenario is merely suggestive, future studies should aim at linking our observation with the new concept of neurotrophin-mediated inhibition of microglial MHC class II expression.

In summary, we demonstrate a vagal-mediated pathway of microglial activation in response to peripheral inflammation, in the area postrema. Vagotomy blocks the up-regulation and induction of microglial expression of MHC class II that accompany colitis. We propose the involvement of the newly

identified concept of neurotrophin-mediated control of microglial MHC class II expression as a possible mechanism regulating microglial activation in response to peripheral inflammation.

CHAPTER 6

THE EXPRESSION OF MONOCYTE CHEMOTACTIC PROTEIN-1 IN THE CNS FOLLOWING COLITIS

6.1 Objective # 4

Determine whether the activation of microglia in response to colitis is preceded by the expression of chemotactic signals (Specifically, the chemokine monocyte chemotactic protein-1 (MCP-1)).

6.2 Background

In most studies of the CNS response to injury or peripheral immune challenge, it is virtually impossible to distinguish between activated resident microglia and infiltrating monocytes. This is due to the lack of a marker, specific to either cell type. In our case, there are two possibilities for the accumulation of MHC class II expressing cells in the area postrema: a) peripheral signals could circulate through the blood, enter this region, and directly activate resident microglia or b) activated monocytes can migrate and colonize the area postrema. The migration of monocytes into the CNS is generally observed during development but also in response to pathological or experimentally induced brain insults. The infiltration of the adult brain by blood-derived monocytes occurs in response to diverse CNS insults, including viral infection (33), tumor growth (93) and experimental injuries (79), and is involved in parenchymal inflammation. Recent studies, mainly performed on peripheral tissues, have shown (*in vitro* and *in vivo*) the existence of the chemokines as a novel class of cytokines, and have implicated their involvement in the mechanism of cell

migration into neoplastic tissues (3). The production of MCP-1 by microglia was demonstrated in a model of rat brain injury (37). Hence, microglial migration as well as monocyte infiltration to the brain could be mediated, in part by the production of MCP-1. Although, peripheral inflammation is neither identical nor similar to brain injury, the mere capacity of resident macrophages to produce (*in vivo*) a powerful chemoattractant as MCP-1, implicates its potential to mediate macrophage/ microglial migration under less traumatic conditions. An additional support for the potential of chemokines in mediating microglial migration into the CNS stems from a study that illustrated the expression of receptors for chemokines on microglia (40).

Further support for the role of central expression of chemokines, in attracting immune cells to the CNS, came from observations of brains of mice that over express the chemokine MCP-1 (29). These mice developed perivascular inflammation at three weeks of age, however, following peripheral (i.p.) injection of lipopolysaccharide, a massive monocyte infiltration of the CNS was noticed. This data indicated that over expression of a chemokine such as MCP-1 could prime the CNS for response to peripheral immune stimuli. Moreover, in models of traumatic brain inflammation, the expression of MCP-1 was upregulated at time points prior to macrophage entry into lesions (35). Although, it is not known whether peripheral inflammation leads to a chemokine mediated increase in monocytes in the CNS, it is clear that central expression of MCP-1 could result in infiltration of monocytes. To establish whether the

activation of microglia in response to colitis is accompanied by the production of chemotactic signals we hypothesized that following colitis microglial activation in the area postrema is preceded by the expression of the chemokine monocyte chemotactic protein-1 (MCP-1).

6.3 Hypothesis

Microglial activation in response to colitis is preceded by the expression of the chemokine monocyte chemotactic protein-1 (MCP-1).

6.4 Methods

Three groups of male Wistar rats (n=4-6; 150-180g) were treated with TNBS or saline, killed, perfused, and their brains sliced, as previously described (in methods of section 1). Additional control groups (each n=2) were treated with ethanol or saline and examined in early and late time points. As all these groups expressed this chemokine weakly and to the same extent, the 12h post saline treatment group served as a control group for the entire TNBS treated groups. Immunohistochemistry was employed for detection of the chemokine MCP-1. An early (12h) and late (72h) time points, following treatment, were examined. Labeled slices were mounted onto slides, and examined in a florescence microscope. Counts were performed in regions where MHC Class II and c-fos expression were induced in response to colitis. To control for the effect of the

extent of colonic inflammation on the expression of MCP-1 in the area postrema, an additional group was pretreated with the anti-inflammatory agent dexamethasone (2mg/Kg), and macroscopic (damage score) as well as microscopic assessment of inflammation (MPO activity assay) were compared to TNBS and saline controls. The expression of MCP-1 in the area postrema was compared to its expression in another circumventricular organ the OVLT. The specificity of the primary antibody was indicated by the lack of staining following preabsorption with the peptide MCP-1 (Serotec).

6.5 Results

Colitis was assessed and revealed the same macroscopic characteristics as explained in the previous chapters. Both at early (12 h) and late (72 h) time points following TNBS treatment, colons were severely damaged (with the respective average damage scores of 7 ± 1.41 SD and 5.5 ± 1.3 SD). In contrast, ethanol and saline treated control groups had little or no damages at all times (at 12 h and 72 h following treatment, the ethanol group had a respective average damage score of 2 and 1 and the saline group had a score of 0).

Twelve hours following colitis, MCP-1 expression increases in the area postrema, and remains elevated at 72 hours (Fig. 6.2). In the dorsal vagal complex, the immunoreactive cells were located in the area postrema (Fig. 6.1). The expression of MCP-1 in the OVLT remained unchanged, and was similar in the TNBS and saline treated groups. All control groups had a low basal level of

MCP-1 immunoreactivity in the area postrema. Two ethanol and two saline treated groups (n=2) were examined at early (12h) and late (72h) time points, and had identical low immunoreactivity levels, hence the 12h saline control group (n=4) was used as a universal control in this experiment. The dexamethasone (2mg/kg) pretreated group, which received TNBS treatment and then killed perfused 72h after treatment, had a significant reduction in MCP-1 immunoreactivity in the area postrema and no effect in the OVLT. The levels of MCP-1 expression in this group were comparable to the control group. MCP-1 expression was examined in other brain structures, in the cerebral aquaduct, which is adjacent to the dorsal vagal complex, levels of expression were elevated, and the labeled cells had a similar morphology to ependymal cells. In the choroid plexus and parenchyma (data not shown) MCP-1 expression was constitutive and unchanged both at early (12 h) and late (72 h) time points following TNBS treatment.

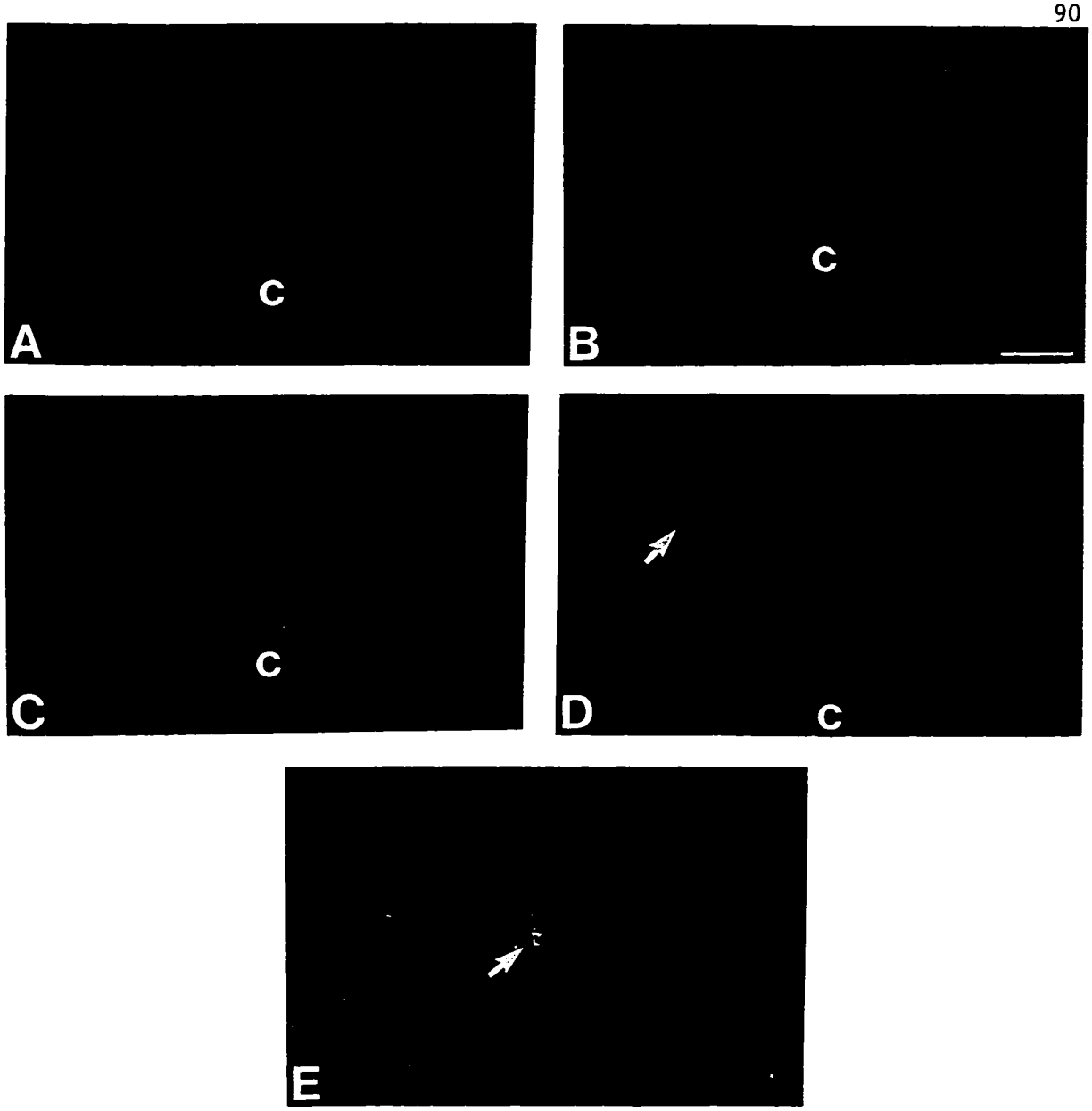

Fig. 6.1

Figure 6.1

Fluorescence micrographs of coronal sections stained for MCP-1 in the dorsal vagal complex from saline (A) and TNBS (B) treated animals at 12 h and TNBS treated (D) and dexamethasone pretreated (C) animals at 72 h following treatment. The appearance MCP-1 immunoreactive cells was absent from the saline treated (A), weak in the dexamethasome pretreated (B), and obvious in both early (12 h) and late (72 h) time points of TNBS treated animals. A higher magnification of a cell expressing MCP-1 taken at 12 h following TNBS treatment is displayed in the bottom micrograph. Scale bar: A-D=100μm; E= 25μm.

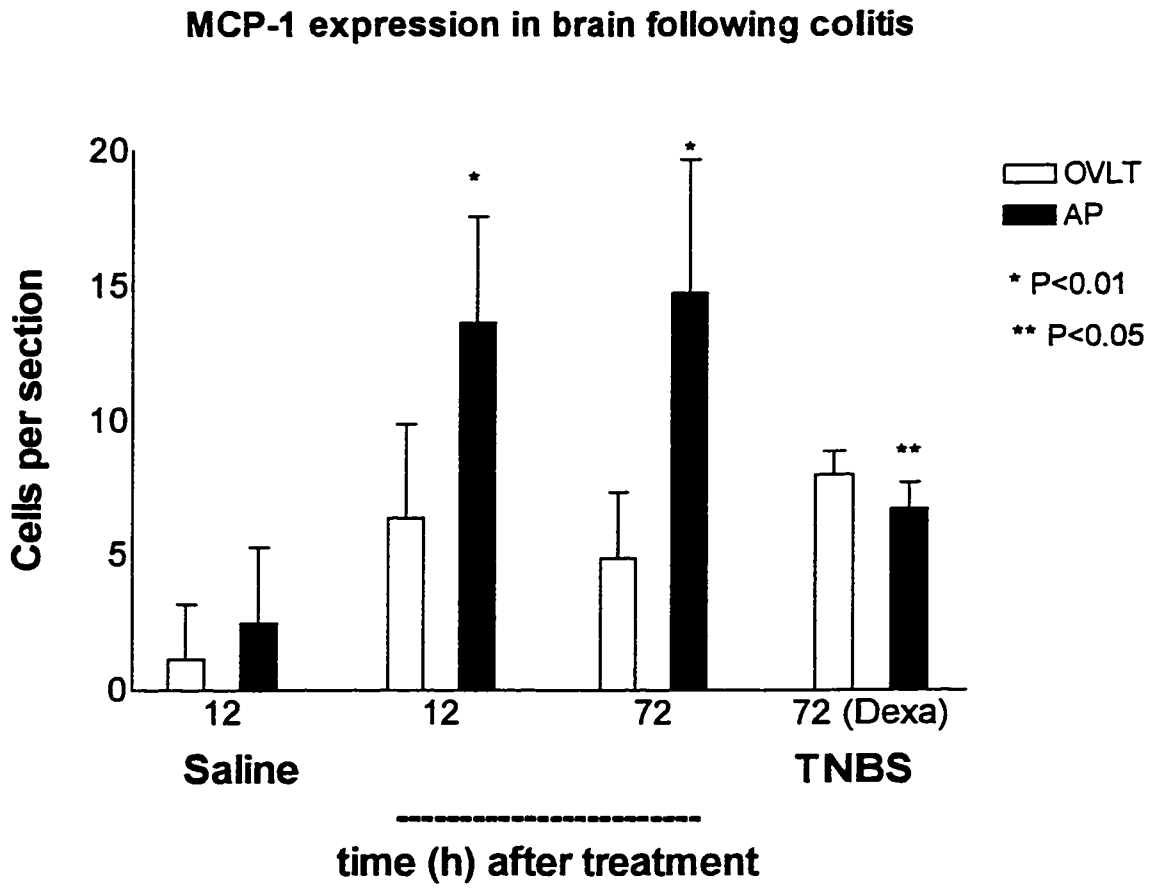

Figure 6.2. MCP-1 expression in brain following colitis. * P<0.01 was obtained for the saline treated group and ** P<0.05 was obtained for the dexamethasone pretreated control group (One way ANOVA). Bars are represented as Mean ± SD.

6.6 Discussion

These data demonstrate the expression of immunoreactive MCP-1 in the area postrema prior and parallel to the appearance of an increased number of microglia expressing MHC class II antigen. The induction of MCP-1 expression following TNBS treatment was compared to saline and ethanol controls that exhibit no immunoreactivity for this chemokine, in the examined regions and the entire brain. Normal rat brains had low constitutive levels of immunoreactive MCP-1 expression in the meninges and choroid plexus. At 12 h after the induction of colitis, the cells expressing this chemokine appear to have a morphology characteristic of ramified microglia. However, due to previous evidence, pointing to astrocytes as being the major source of MCP-1 in the CNS in response to peripheral administration of endotoxin (36), these observations should be interpreted with caution, and future studies should aim at identifying the specific cell type expressing MCP-1 in response to colitis. Following peripheral administration of endotoxin, the expression of MCP-1 is seen only at early time points (peaks at 4-8 h and declines at 24 h), however, in the hapten-induced model of colitis, immunoreactive MCP-1 is expressed in the area postrema both at early (12 h) and late (72 h) time point after treatment. These data suggests that the chemokine MCP-1 could be involved in recruiting microglia or circulating monocytes from the meninges or blood, respectively, to the area postrema. This introduces a new possible role for this chemokine in regulating the central distribution of MHC class II expression in response to

peripheral inflammation.

CHAPTER 7

INTERLEUKIN-1β IMMUNOREACTIVITY IN THE IN THE CNS FOLLOWING COLITIS

7.1 Objective # 5

Examine the production of the cytokine interleukin-1β in the region where microglia are activated upon the induction of colitis.

7.2 Background

A wide variety of CNS responses to peripheral immune challenge have been shown to be mediated by the central action of IL-1β, these include a) increased body temperature (49), b) elevation of plasma levels of corticosterone (28), c) changes in sleep pattern and behavior (26), and d) alteration in brain neurotransmission (60). During peripheral inflammation, IL-1β is produced and released by activated immune cells, however, very small amounts of this large hydrophilic molecule are able to cross the blood-brain barrier (BBB)(89). Several groups have shown that following the onset of immune stimulation, the gene encoding for the cytokine IL-1β is expressed in elevated amounts in the CNS first in areas that lack a BBB, such as the area postrema (100), and subsequently within brain parenchyma in areas that have intact BBB, such as the paraventricular and arcuate nuclei of the hypothalamus (105). Further examination has revealed that the cellular source of IL-1β mRNA expression in the CNS, induced by peripheral inflammation, is primarily glial (82). The induction of IL-1β mRNA expression, in circumventricular organs, occurred at time points (6-10h after peripheral endotoxin administration) characterized by

the appearance of microglial cells that were immunoreactive for this cytokine. Hence, endotoxin-induced peripheral inflammation is superseded by the central expression of IL-1β both at the mRNA and protein levels.

Another relevant issue is the role of the vagus nerve in mediating CNS production of IL-1β in response to peripheral endotoxin or IL-1β. A number of studies have demonstrated that vagotomy blocks the central expression of IL-1β, and attenuates the related symptoms and behavioral effects, induced by the above stimuli (see the Introduction and Ref. (10)). In the same manner, the central (i.c.v.) administration of IL-1 receptor antagonist was shown to abrogate the effects of these stimuli (8) suggesting that following peripheral inflammation the vagus nerve carries immune signals from the abdomen (innervated by vagal afferent nerves) to the CNS leading to the central production IL-1β. The route of administration of the immune activator was crucial in all of these studies as only the effects mediated by intraperitoneal (and not intravenous) administration of either lipopolysaccharide or IL-1β were attenuated by vagotomy. Since, vagal branches which are higher than the diaphragm remained intact, they were still capable of sensing systemically administered immune activators, and this provides a probable explanation for the discrepancy between the different routes of administration of the immune stimulator.

Taken together, these findings illustrate the role of the vagus nerve in mediating the central production of IL-1β, in response to peripheral immune stimuli. Colonic inflammation involves the production of many cytokines,

including IL-1β, which can interact with vagal afferent nerves. Since both colitis and intraperitoneal administration of endotoxin result in the abdominal production of cytokines such as IL-1β, we hypothesized that the induction of colitis is followed by the central expression of IL-1β

7.3 Hypothesis

The expression of IL-1β is induced in regions where microglia are activated in response to colitis.

7.4 Methods

Six groups of male Wistar rats (n=3; 150-180g) received either TNBS or saline treatment and subsequently killed and perfused at three consecutive time points (4, 8, and 12h). To assess the severity of colitis colons were removed, macroscopic damage scored, and samples taken for histological examination and MPO activity assay. The perfusion was slightly modified (as explained previously), rats were perfused and incubated for 1h in 2% paraformaldehyde, and then transferred to a sucrose (20% in PBS) solution for overnight incubation. Brain sections were frozen and cut to 20μm coronal sections and thaw mounted onto gelatin-coated slides. Immunohistochemistry was used to detect the cytokine IL-1β. The cytokine specific primary antibody was used according to a protocol provided by the supplier (R&D systems). For positive staining control, a

previous study (101) was repeated where a small group of rats (n=2) were injected intraperitoneally with lipopolysaccharide (2 mg/Kg), and there brains examined under a light microscope. Due to the lack of distinct morphology of the labeled cells, it was not possible to quantify the number of cells expressing this cytokine, hence the analysis of IL-1β expression was exclusively qualitative. Preabsorption of the primary antibody with the peptide IL-1β resulted in the lack of staining, indicating specificity.

7.5 Results

At all of the early time points (4, 8, 12 h) following TNBS treatment, the macroscopic assessment of colonic damage revealed severe ulcerations (with the respective average damage scores of 5.7 ± 2; 7.3 ± 1.2; and 7 ± 1.4). The saline treated control groups had no colonic ulcerations or macroscopic damage, at any time point examined (4, 8, 12 h). In saline-treated rats, there was no IL-1β-like immunoreactivity present in the brain at any time point examined. Nonetheless, at 4 h after the induction of colitis, IL-1β immunoreactivity was detected at the border of the area postrema and the fourth ventricle, and the adjacent meninges. Immunoreactive cells also appeared in the choroid plexus and parenchyma at the level of the brainstem and anterior brain (not shown). In the meninges lining the area postrema, the immunoreactivity for IL-1β decreased at 8 h and reappeared at 12h after TNBS treatment. Moreover, at 12 h after TNBS treatment, microglia-like cells appeared within the area postrema, and

immunoreactivity reappeared in the meninges lining the area postrema (Fig. 7.1). Consistent with previous literature (101), brain sections, taken from the endotoxin-treated positive control group, had an increase in the intensity of staining in the area postrema and macrophage-like immunoreactive cells in the choroid plexus. At 8 h and 12 h after the induction of colitis, IL-1β immunoreactive cells with macrophage or microglial morphology appeared in the meninges and in the adjacent cerebral cortex, once more in line with previous literature. Although, most immunoreactive regions and cells had no consistent and well defined structure, some labeled cells, which appeared 12 h following TNBS treatment, had microglial morphology with a limited number of spiny protrusions that were less subdivided than the characteristic microglial ramifications.

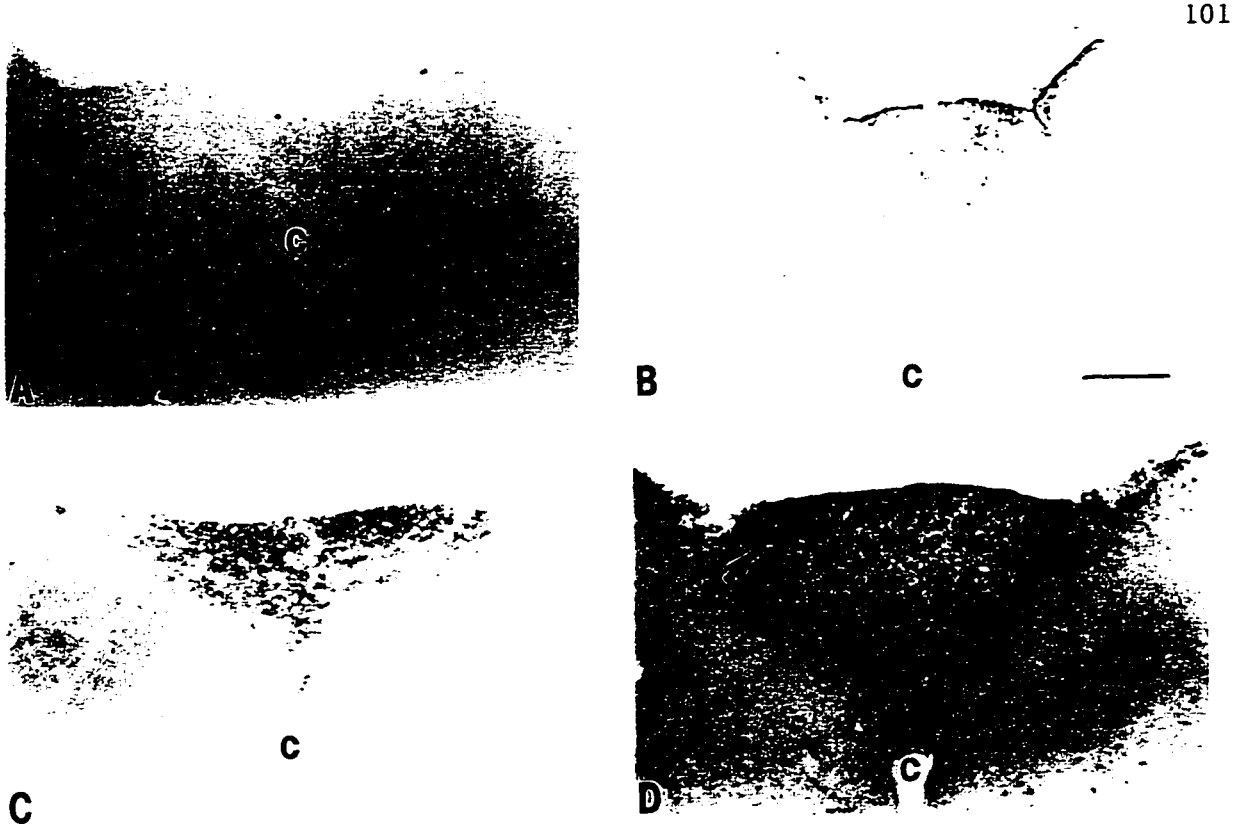

Fig. 7.1

Figure 7.1.

Photomicrographs of IL-1β immunoreactivity in coronal sections of the dorsal vagal complex at multiple early time points (4, 8, 12 h) after the induction of colitis. (A) No staining of IL-1β immunoreactive cells was present in a normal animal. (B) Some staining for IL-1β was detected at the border of the area postrema and the fourth ventricle at 4 h following colitis. (C) IL-1β immunoreactivity in the area postrema decreased at 8 h compared to 4 h following TNBS treatment, and (D) reappeared in cells with microglial morphology at 12 h post colitis. Arrow in D is pointing to an immunoreactive cell on the border of the AP and NTS. c, central canal. Scale bar: A-D=100μm.

7.6 Discussion

In this study, we demonstrate the appearance of IL-1β immunoreactivity in response to hapten-induced colitis, which was not specific to the area postrema and was found in other CNS regions. In the area postrema, the staining intensity was low and restricted to the surface lining the forth ventricle at 4 h after the induction of colitis, and was maximal and present within the area postrema at 12 h after treatment. At 4 h after TNBS treatment, IL-1β immunoreactivity appeared also in the choroid plexus and the meninges, at the level of the brainstem and anterior brain. This observation is in line with a previous study (101), that demonstrates the induction of immunoreactive IL-1β expression in response to peripheral endotoxin at various brain sites, including circumventricular organs. It has been suggested that following peripheral administration of lipopolysaccharide, IL1-β is produced by peripheral macrophages or monocytes, acts on endothelial cells expressing its receptor (type I), and induces its own expression in the CNS via an unknown second messenger (possibly nitric oxide (59)). Both the endotoxin and hapten-induced colitis models of peripheral inflammation are accompanied by the production of many proinflammatory cytokines, including IL-1β. Therefore, we suggest that the appearance of IL-1β immunoreactive cells in the CNS in response to colitis is not region-specific and is probably a general feature of models of peripheral inflammation.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this study was to examine the response of the CNS to peripheral inflammation in the hapten-induced model of colitis. Unlike in other various models of peripheral inflammation, the hapten-induced model of colonic inflammation is regulated by a combination of neuronal and immunological factors. The extrinsic innervation of the colon is comprised of afferent sensory nerves that transmit neuronal signals to the brain, which constantly processes the incoming information, and regulates peripheral homeostasis, via efferent neuronal projection. In addition, the rich and diverse composition of immune cells that reside in the colon and orchestrate the initiation, progression, and termination of colitis, have the capacity to produce and secrete potent cytokines that could be involved in periphery to brain signaling, via circumventricular organs. Due to these features of colitis, our objectives were to:

1. Examine the activation of neurons in the dorsal vagal complex that receives and processes afferent sensory input from the colon.
2. Demonstrate the activation of microglia in response to colitis, in the dorsal vagal complex, which integrates peripheral and blood-borne signals.
3. Establish whether microglial activation in response to colitis is mediated by a neural route of communication (via the vagus nerve) or by a blood-born route (preferentially in CVOs).
4. Determine whether microglial activation in response to colitis is accompanied by the expression of chemotactic signals.
5. Examine the central appearance of immunoreactive IL-1β in the dorsal vagal

complex, in response to colitis.

First, we hypothesized that following colitis, sensory nerves would carry signals from the inflamed colon to the dorsal vagal complex, leading to neuronal activation (by detecting Fos expression). Based on the lack of double-labeling with microglial (OX-42) and astrocytic (GFAP) specific primary antibodies, we concluded that Fos expressing cells were probably neurons. Our finding demonstrated the differential induction of c-fos expression within two sites in the dorsal vagal complex, following colitis. In the NTS, the expression of Fos was induced by ethanol alone, whereas Fos expression in the area postrema depended on the combined administration of the hapten and ethanol. As the intrarectal administration of either TNBS or ethanol alone did not induce colonic inflammation or Fos expression in the dorsal vagal complex, we suggested that neuronal activation in the area postrema might have depended on the summation of neuronal and blood-borne signals, derived from the inflamed colon. These signals could have reached the area postrema via the NTS, which receives sensory colonic input, or the fourth ventricle that allows the free passage of blood-borne cytokines or neuropeptides. As hapten-induced colitis involves the production of the potent microglial activator IFN-γ, we hypothesized that microglia are activated in response to colitis.

After careful examination of the dorsal vagal complex and two other CNS regions, such as the OVLT and the hippocampus, we observed microglial activation within the area postrema, at 72 h following colitis. Our observation

was specific to the area postrema and did not appear in other sites at the level of the dorsal vagal complex or the other two CNS regions examined. Moreover, pretreatment with the anti-inflammatory glucocorticoid dexamethasone abolished the activation of microglia in response to colitis, indicating that this phenomenon correlated with the severity of colonic inflammation. A further support for our hypothesis came from the use of another experimental model of colitis, the IL-10 deficient mice model. In this model, we found a change in the distribution and number of activated microglia (which was not statistically significant) in the area postrema of mice, two to three weeks before they develop severe enterocolitis. Although this finding is preliminary, it provides initial support for the involvement of IL-10 in the *in vivo* regulation of microglial antigen presentation. Future, studies should aim at establishing a full time-course for brainstem immunoreactive MHC class II expression in IL-10 deficient mice before and during the onset of enterocolitis and whether it correlates with the severity of colonic inflammation. Other future studies could attempt to administer IL-10 centrally and observe its effect on microglial activation in response to colitis.

As our finding was confined to the area postrema, we then proposed that the extensive projections between the area postrema and the vagus nerve could account for the region specific appearance of activated microglia, and hypothesized that microglia activation in response to colitis is mediated by the vagus nerve. The quantitative examination of activated microglia in the area postrema of vagotomized rats, revealed that vagotomy abolished the increase in

microglial activation and MHC class II expression in response to colitis, however, it impaired the normal steady-state of MHC class II expression within the dorsal vagal complex. In both inflamed and non-inflamed vagotomized rats, microglia were activated in the NTS and the dorsal motor nucleus of the vagus. This finding is in line with a previous study (84) that demonstrated microglial induction of MHC class II expression in response to vagotomy alone. As these nuclei were devoid of microglial expression of MHC class II in the non-vagotomized animals, we suggested that this phenomenon was a feature of the operating procedure. These data introduced for the first time evidence for a vagal mediated cellular activation. The concept that the vagus nerve could mediate microglial activation in response to peripheral inflammation is in line with other studies demonstrating vagal mediated periphery to brain signaling in immune challenged animals (58). At present, the *in vivo* mechanisms controlling microglial antigen presentation in the CNS are unknown, however, a recent study has strongly supported the role of neurotrophins in maintaining low MHC class II expression by microglia (73). In this study, the addition of NGF, BDNF, or NT-3 to mixed explant cultures resulted in the inhibition of IFN-γ induced microglial MHC class II expression. The blockade of the p75 neurotrophin receptor (with antibodies) attenuated this effect, suggesting that these neurotrophins act via a specific receptor. The significance of this is that active neurons have the capacity to inhibit microglial expression of MHC class II antigen, and that modulation in the extracellular neurotrophin content could serve as a mechanism for microglial activation in the

CNS. In light of another study (41) demonstrating the accumulation of these neurotrophins in vagal afferent neurons, we propose that the appearance of activated microglia in the vicinity of the NTS and dorsal motor nucleus of the vagus was the result of the lack of secretion of neurotrophins in to the extracellular matrix by degenerating neurons.

Due to the inability to distinguish between infiltrating monocytes, parenchymal microglia, and local resident activated microglia, our next aim was to establish whether the increase in the appearance of activated microglia in the area postrema was assisted by chemotactic signals. This was supported by a two separate studies demonstrating astrocytic expression of MCP-1 in response to peripheral administration of endotoxin and an increased monocytic cell population in endotoxin treated transgenic mice overexpressing the chemokine MCP-1. We report for the first time the appearance of MCP-1 immunoreactive cells in the area postrema in response to colitis. This immunoreactive appearance depended on the extent of colonic inflammation, as it was abolished by pretreatment with dexamethasone. Although the morphological examination of MCP-1 immunoreactive cells suggests a structure similar to the ramified microglia, previous studies have shown that MCP-1 is produced in the CNS primarily by astrocytes. Future studies should aim at establishing the cellular source of MCP-1 expression in response to colitis.

Finally, our last task was to examine the central production of IL-1β in the area postrema, where microglia are activated in response to colitis. Due to

previous data showing the early appearance of this cytokine in circumventricular organs in response to peripheral endotoxin (101), we focused on early time points following colitis (4, 8, 12 h). Unlike the region specific expression of microglial and neuronal activation markers in the area postrema, IL-1β immunoreactivity appeared at 4, 8, and 12 h in multiple CNS regions. First it was present in the meninges and choroid plexus and then in the brain parenchyma at the level of brainstem and anterior brain. The inconsistent morphology of the labeled cells prevented us from quantifying the increases in the immunoreactive expression of this cytokine. Because, the immunoreactive appearance of this cytokine was not region specific, we suggested that it was a general feature of the CNS response to the onset of peripheral inflammation. A current hypothesis (59) suggests that, following peripheral administration of lipopolysaccharide, IL-1β is produced by peripheral macrophages, acts on endothelial cells expressing its receptor (type I), and induces its own expression in the CNS via an unknown second messenger. The peripheral endotoxin and hapten-induced colitis models of peripheral inflammation lead to the peripheral production of multiple cytokines, neuropeptides, and other yet unknown factors that might signal the brain to produce IL-1β. One possible second messenger is nitric oxide, as the inducible form of its enzyme inducible nitric oxide synthase has been shown to be expressed in brain vasculature in response to peripheral immune challenge (104).

A question, which arises from the above data, is whether activated

microglia serve a function in the regulation of peripheral immune responses and if they do, by which specific signals. One can argue that peripheral immune signals can mimic the effects of direct CNS insults and thus prime microglia for antigen presentation, however, in this case we would expect the activation of microglia in all circumventricular organs following inflammation. In our study, microglial activation was found to be specific to the area postrema, which lies in the dorsal vagal complex. This brain structure is known to be involved in homeostatic functions via either direct innervation (through the dorsal motor nucleus of the vagus) of the viscera or projections that terminate in the hypothalamus and lead to the activation of the HPA axis. Hence, we suggest that activated microglia partake in the generation of central signals that control peripheral homeostasis. Future studies should aim at identifying these relevant signals, which could be a part of the already known repertoire of microglial products. For example, the central production and action of many of the microglial derived cytokines (IL-1β and TNF-α) have been shown to have a role in the homeostatic regulation of peripheral immune responses, however, the composition and central action and function of microglial derived cytokines in response to peripheral immune insults remain to be determined.

In summary, we have demonstrated various aspects of the response of the CNS to peripheral inflammation in the model of colitis. At 12h following the induction of colitis, neurons are activated and immunoreactive MCP-1 is expressed in the area postrema. Between 4 to 12 h, IL-1β immunoreactive cells

with faint microglial morphology appear throughout the choroid plexus, meninges, and at multiple levels of brain parenchyma. This is followed by a possibly vagal mediated activation of microglia in the area postrema, at 72 h following colitis. However, the significance of this study is not clear, and future studies should attempt to establish whether activated microglia function in the CNS homeostatic regulation of peripheral immune responses, and if so, how they participate in this task. As these events occur in sites that are anatomically related to the HPA axis, microglia have the potential to produce potent signals capable of interacting with the periphery from within the CNS.

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IMAGE EVALUATION
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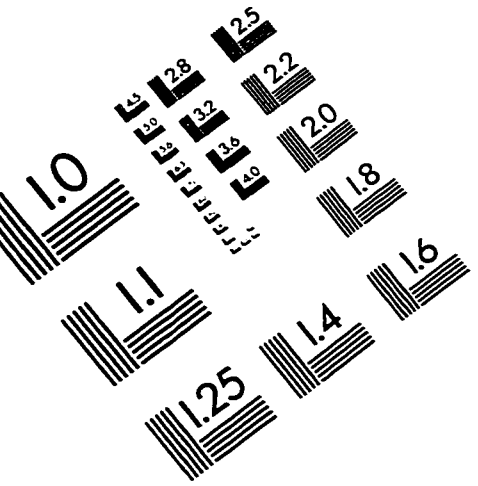

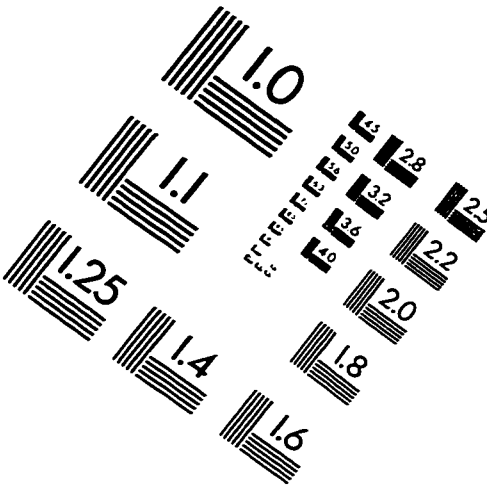

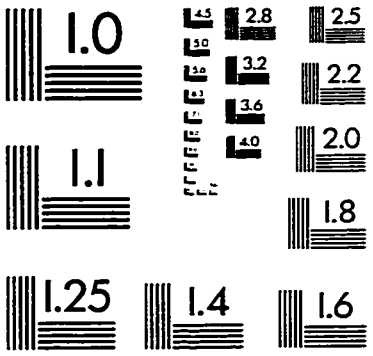

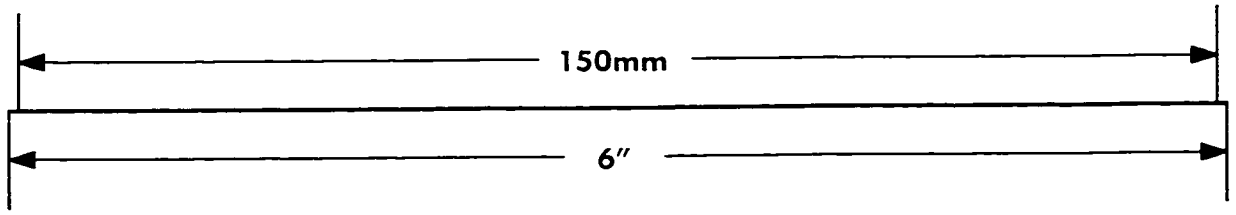

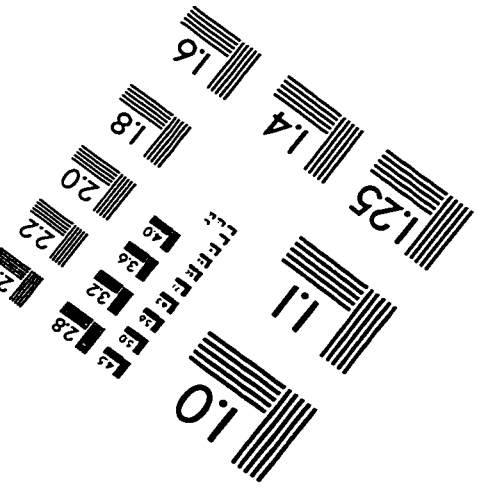

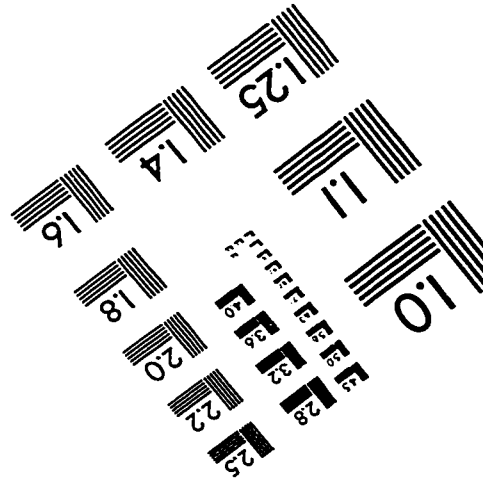

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